EFFECT OF BEEF CONSUMPTION ON HDL DENSITY DISTRIBUTION AND FUNCTIONALITY IN HEALTHY INDIVIDUALS

A Dissertation

by

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ABSTRACT

The adverse health associations reported for red meat may be attributed to increased saturated fatty acid (SFA) intake. This project aims to evaluate the effects of beef fatty acid composition and lean beef on atherosclerotic cardiovascular disease (ASCVD) risks in two healthy populations.

Study 1:

Twenty-five normocholesterolemic men consumed in random order: 5-114 g, 24% fat beef patty/wk for 5 wk of both low (M:S = 0.71) and high monounsaturated fatty acid (MUFA) (M:S = 1.10) beef patties (~40 EN% fat diet). Increased beef consumption had no effect on circulating adipokines, homocysteine, paraoxonase1 (PON1) activity, HDL density distribution and HDL_3 apolipoprotein composition. Independent of fatty acid composition, beef consumption reduced HOMA-IR scores and the pro-inflammatory 5-keto-eicosatetraenoic acid (5-KETE) in HDL (P <0.05). The changes were positively correlated with baseline HDL-C and negatively correlated with baseline HDL EPA status respectively (P <0.05). Overall beef consumption increased HDL_3 sphingosine-1-phophate (S1P) (P <0.05) and this change was negatively correlated with baseline triglyceride, total cholesterol, LDL-C and HDL_3 ApoE (P <0.01).

Study 2:

Twenty-seven mildly hypercholesterolemic subjects consumed in random order: Healthy American diet (HAD), Dietary Approaches to Stop Hypertension (DASH), Beef

in an Optimal Lean Diet (BOLD) and BOLD plus extra protein (BOLD+) (20, 28, 113, 153 g lean beef/d, respectively, all diets ~30 EN% fat) for 5 wk. The BOLD+ diet reduced absolute and fractional abundance of small LDL_4 (P <0.05) relative to HAD. Baseline HOMA-IR was strongly associated with LDL_4 responsiveness to the DASH (P = 0.003) and BOLD diets (P = 0.018). LDL_4 decreased after the BOLD (P = 0.003) and BOLD+ (P = 0.046), but not DASH (P = 0.069) diets in the low baseline HOMA-IR group. The high baseline HOMA-IR group tended to show favorable changes in LDL_4 only after the BOLD+ diet (P = 0.078) with a significant dietary effect observed (P = 0.013).

Our studies showed that individual response to beef intervention, regardless of beef fatty acid composition or total fat, is influenced by baseline health status. In healthy individuals, increased beef consumption may have a favorable effect on ASCVD risk factors.

DEDICATION

This work is dedicated to my beloved parents, Xingyi Wang and Weizhou Wu.

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NOMENCLATURE

5-HEDH	5-Hydroxyeicosanoid Dehydrogenase
AA	Arachidonic Acid
ATF3	Activating Transcription Factor 3
ABCA1	ATP Binding Cassette Transporter A1
ABCG1	ATP Binding Cassette Transporter G1
ALA	α-Linolenic Acid
ASCVD	Atherosclerotic Cardiovascular Disease
AUC	Area Under Curve
BOLD	Beef in an Optimal Lean Diet
CAD	Coronary Artery Disease
CE	Cholesterol Ester
CETP	Cholesteryl Ester Transfer Protein
CHD	Coronary Heart Disease
COX	Cyclooxygenase
CRP	C-Reactive Protein
CUDA	1-Cyclohexyl-Ureido-3-Dodecanoic Acid
DASH	Dietary Approaches to Stop Hypertension
DGLA	Dihomo-γ-Linolenic Acid
DHA	Docosahexaenoic Acid
DiHOME	Dihydroxyoctadecenoic Acid

EFA	Esterified Fatty Acid
EKODE	Epoxyketooctadecenoic Acid
eNOS	Endothelial Nitric Oxide Synthase
EPA	Eicosapentaenoic Acid
EpOME	Epoxyoctadecenoic Acid
FCR	Fractional Catabolic Rate
FTR	Fractional Transfer Rate
HAD	Healthy American Diet
HDL	High-Density Lipoprotein
HETE	Hydroxyeicosatetraenoic Acid
ICAM-1	Intercellular Adhesion Molecule 1
KETE	Ketoeicosatetraenoic Acid
KODE	Ketooctadecadienoic Acid
LA	Linoleic Acid
LCAT	Lecithin:Cholesterol Acyltransferase
LOX	Lipoxygenase
LP	Lipoprotein
LPL	Lipoprotein Lipase
LT	Leukotriene
MCP-1	Monocyte Chemotactic Protein 1
MRM	Multiple Reaction Monitoring
MUFA	Monounsaturated Fatty Acid

NCEP	National Cholesterol Education Program
PAFAH	Platelet-Activating Factor Acetylhydrolase
PAI-1	Plasminogen Activator Inhibitor 1
PG	Prostaglandin
PL	Phospholipid
P-OM3	Omega-3 Polyunsaturated Fatty Acid Ethyl Ester
PON	Paraoxonase
PTX3	Pentraxin 3
PUFA	Polyunsaturated Fatty Acid
RCT	Reverse Cholesterol Transport
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
SAA	Serum Amyloid A
SCD	Stearoyl-CoA Desaturase
sdLDL	Small Dense Low-Density Lipoprotein
sEH	Soluble Epoxide Hydrolase
SFA	Saturated Fatty Acid
SR-BI	Scavenger Receptor Class B Type I
TGFβ	Transforming Growth Factor β
TRL	Triglyceride-Rich Lipoprotein
TX	Thromboxane
UPLC	Ultra Performance Liquid Chromatography

VAP	Vertical Auto Profile
VCAM-1	Vascular Cell Adhesion Molecule 1
VSMC	Vascular Smooth Muscle Cell

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

The inverse association of high-density lipoprotein cholesterol (HDL-C) concentration with atherosclerotic cardiovascular disease (ASCVD) is well established in clinical and epidemiological investigations. The Framingham Heart Study and the Prospective Cardiovascular Münster Study showed that for every 1.0 mg/dL increase in HDL-C, ASCVD risk decreases by 2-3% (1). In the National Cholesterol Educational Program Adult Treatment Panel III (NCEP ATP III) guidelines, a HDL concentration of <40 mg/dL is defined as an independent risk factor for ASCVD (2). As a result, increasing attention has been focused on raising HDL-C concentration as a strategy to prevent ASCVD. However, large clinical trials to increase HDL-C through pharmacologic agents show no clear ASCVD risk reduction with HDL-C concentration increases. A meta-analysis of 9,959 subjects drawn from niacin trials showed that elevation in HDL-C by niacin was not associated with reduced ASCVD risk (3). In large clinical trials, the improvement in HDL-C by cholesteryl ester transfer protein (CETP) inhibitor torcetrapib also failed to show preventive effects on atherosclerotic progression (4). Further, the recent dal-ACUTE trial demonstrated the dissociation between pharmacologically induced elevation in HDL-C and HDL protective function in acute coronary syndrome patients (5). In plasma, HDL particles serve as a platform on which rapidly to assemble and disassemble proteins and lipids. Accumulating evidence showed

that inflammation and oxidative stress are crucial modulators of HDL proteome and lipidome (6-8). The inability of HDL-C raising therapies have shed new insights into the complexity of HDL composition and function. Consequently, the research focus on HDL-C has now started shifting towards alternative metrics of HDL such as particle size, subclass distribution, and HDL functionality.

Disease development and HDL functionality

Atherosclerosis is an inflammatory process that is initiated in part by the presence of oxidized LDL in the artery wall, followed by endothelial dysfunction, recruitment of monocytes and formation of foam cells (9). Historically, the athero-protective potential of HDL is attributed to its role in reverse cholesterol transport (RCT). This process promotes cholesterol efflux from peripheral tissues and most importantly, macrophage cells and returns cholesterol to liver to be metabolized or reutilized. As the HDL research moves increasingly toward functionality, other important athero-protective mechanisms have been uncovered. HDL can prevent the generation of reactive oxygen species (ROS), reduce the oxidation of phospholipids and inhibit the pro-inflammatory actions of minimally modified LDL. The anti-oxidant property of HDL is mostly attributed to its anti-oxidant enzymes including paraoxonase (PONase), ApoA-I, and platelet-activating factor acetylhydrolase (PAFAH) (10, 11). For anti-inflammatory protection, functional HDL inhibits the expression of monocyte chemotactic protein 1 (MCP-1) in vascular smooth muscle cells (VSMCs), promotes endothelial cell barrier integrity and prevents the recruitment of pro-inflammatory cells. Many anti-inflammatory activities of HDL have been shown to be mediated by its

sphingosine-1-phosphate (S1P) content and the activation of S1P G-protein coupled receptors (12, 13). There is also evidence that HDL can effectively downregulate Toll-like receptor signaling by disrupting lipid raft formation (14) and by inducing the transcriptional regulator activating transcription factor 3 (ATF3) in immune cells (15). In addition, HDL can promote vasodilation via nitric oxide synthase (NOS) activation (16, 17) and can attenuate apoptotic intracellular signaling in endothelial cells (18).

HDL metabolism

Plasma HDL is a heterogeneous group of small discoid and spherical particles. HDL particles are initially produced as lipid-poor ApoA-I by the liver or intestine (19) and immediately lipidated via ATP binding cassette transporter A1 (ABCA1) mediated cholesterol and phospholipid (PL) efflux, forming discoidal nascent pre-β HDL (20, 21). Phospholipids from lipoprotein lipase (LPL) hydrolyzed triglyceride-rich lipoproteins (TRL) present another important lipid source for nascent HDL (22). These nascent HDL particles are excellent substrates for lecithin:cholesterol acyltransferase (LCAT), that esterifies a fatty acid from phosphatidyl-choline (a.k.a. lecithin) to cholesterol to form cholesterol ester (CE) and lysophosphatidyl choline (23). Cholesterol esters are highly hydrophobic and migrate into the core of HDL, resulting in the core expansion of discoidal HDL and generation of spherical mature HDL. Small spherical HDL, known as HDL_3, further acquires cholesterol via ATP binding cassette transporter G1 (ABCG1) and scavenger receptor class B type I (SR-BI) from peripheral tissues and forms large spherical HDL, known as HDL_2 (24, 25). The particle size and composition of HDL are constantly remodeled by various cell surface transporters, lipid transfer proteins and

lipases. Cholesteryl ester transfer protein (CETP) transfers HDL-C to TRL in exchange for TG and generates TG-rich HDL (26). Phospholipid transfer protein (PLTP) promotes the net transfer of PL from TRL to HDL as well as the exchange of PL between them (27). PLTP also acts as a conversion factor, converting HDL_3 into larger and smaller HDL particles (28). Further, HDL particle size can be reduced through the actions of hepatic lipase (HL), and endothelial lipase (EL) and to a lesser extent, LPL (29). HDL lipids are mainly catabolized in the liver via SR-BI mediated selective uptake (30) or LDL receptor mediated whole particle uptake (31).

Role of HDL components

As the smallest (7-12 nm) and most dense (d = 1.063-1.21 g/mL) lipoprotein class in plasma, HDL has the highest protein (30-70% by weight) and PL (surface molecules, 40-60% of total lipid) content (32). As mentioned above, HDL can exert its athero-protective functionality through multiple mechanisms, mainly including RCT, anti-oxidation, anti-inflammation, vasodilation and cytoprotection. This broad spectrum of HDL functions gives an excellent reflection of the complexity of HDL composition. Recent development of mass spectrometry technology has provided novel insight into HDL proteome and lipidome. Currently over 80 proteins and more than 200 distinct lipid classes have been identified in HDL, many of which have been shown linked to HDL functionality and reflective of ASCVD risk (7, 33).

ApoA-I

ApoA-I, a 28 kDa protein, is the principal protein of HDL, constituting about 70% of their protein content (31). ApoA-I has a highly dynamic structure, containing 8

amphipathic α-helical domains of 22 amino acids each. These helical structures form a hinge domain that allows ApoA-I to switch between different conformations and bind variable amounts of lipids (34, 35). ApoA-I plays a key role in HDL anti-atherogenic function, and is an independent and inverse predictor of CVD (36). ApoA-I can interact with ABCA1, ABCG1 and SR-BI and facilitate RCT by promoting cellular cholesterol efflux, activating LCAT, and forming mature HDL (37). ApoA-I prevents LDL oxidation by removal of seeding phospholipid hydroperoxides from LDL, followed by reduction of hydroperoxides using methionine residues as the reductant (38, 39). ApoA-I also stabilizes other HDL anti-oxidant enzymes such as PON1 (40). ApoA-I and ApoA-I containing reconstituted HDL (rHDL) infusion studies have showed that ApoA-I can inhibit endothelial expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1), and effectively reduces monocyte activation and neutrophil adhesion (41, 42). A study with transgenic mice showed that human ApoA-I diminishes LPS-induced systemic inflammation by suppression of cytokine release and reduction of CD14 expression (43). Moreover, ApoA-I has been identified as the main HDL component that protects endothelial cells from primary apoptosis under oxidative stress (44).

ApoA-II

ApoA-II is the second major protein in HDL, constituting about 20% of HDL total protein. ApoA-II is synthesized by the liver and mainly found in HDL particles containing both ApoA-I and ApoA-II. Unlike ApoA-I, the role of ApoA-II in atherogensis and HDL metabolism has not been definitely established. While ApoA-II is

an efficient acceptor of ABCG1 mediated cholesterol efflux from macrophages (45), HDL isolated from ApoA-II transgenic mice stimulates the production of lipid hydroperoxides in endothelial cells and promotes monocyte migration into the artery wall (46). Overexpression of human ApoA-II in mice can cause ApoA-I and PON1 displacement from HDL particles and resulted in accelerated atherosclerosis (47). Data from clinical and epidemiological studies are similarly inconclusive. Several epidemiological studies have reported that low plasma ApoA-II is associated with increased risk of CHD, including the large scale (10,592 subjects) Prospective Epidemiological Study of Myocardial Infarction Study (48-51). On the other hand, data from the Etude Cas-Témoins de l'Infarctus du Myocarde (ECTIM) study (1,357 subjects) have showed that decreased ApoA-II due to genetic polymorphisms were not associated with CHD (52).

ApoE

ApoE is a key regulator of cholesterol homeostasis in mammals and is readily redistributed between ApoB-containing lipoproteins and HDL. While ApoE only constitutes about 6% of HDL total protein (53), it is structurally similar to ApoA-I (54) and plays critical roles in RCT. For example, lipid poor ApoE promotes cholesterol efflux from ABCA1 pathway and facilitates pre-β HDL maturation into α-HDL (55). HDL ApoE also can interact with the polar head groups of HDL phospholipids and allows easy core expansion after LCAT converts FC to CE (56). Both ABCA1 and ABCG1 are highly expressed in macrophages. In CETP deficient individuals, who have large HDL particles and impaired ABCA1 mediated cholesterol efflux, their HDL_2 has

enhanced ability to promote cholesterol efflux from macrophages in an ABCG1-dependent pathway due to an increase in ApoE content (57). As a potent ligand for LDL-R, ApoE promotes the hepatic uptake of HDL CE via LDL-R (58). It can also interact with SR-BI and promote SR-BI mediated cholesterol efflux and selective lipid uptake by the liver (59). In addition, ApoE confers arthero-protective functions beyond its effect on cholesterol metabolism. One recent study showed that HDL ApoE induced cyclooxygenase-2 (COX2) up-regulation and lipoxygenase (LOX) down-regulation in vascular smooth muscle cells contributes to the suppression of extracellular matrix gene expression and thus the maintenance of arterial elasticity (60).

S1P

S1P is an important signaling molecule that regulates diverse cellular processes involved in immune response. S1P is generated by sphingosine kinase mediated phosphorylation of sphingosine, the direct precursor generated from the ceramide/sphingomyelin metabolic pathway. In plasma, S1P occurs in a concentration of 200-1,000 nM and is mostly carried by HDL (~65%-80%) (61). In blood, S1P predominantly binds to ApoM and to a lesser extent albumin (62). Erythrocytes are the main source of circulating S1P but S1P can also be produced by platelets, vascular and lymphatic endothelium (63, 64). The higher concentration of S1P in serum than in plasma is attributed to S1P release from platelet activation (65).

Numerous S1P depletion or S1P receptor blocking studies showed that many anti-atherogenic functions of HDL are mediated by S1P and the activation of its five G-protein coupled receptors termed $S1P_{1\rightarrow5}$. Nofer et al. (16) found that HDL-S1P

promotes NO production by the eNOS and induces NO-dependent vasodilation in explanted arteries and *in vivo* via $S1P_3$. On the other hand, Wilkerson et al. (66) have showed that HDL-S1P promotes endothelial cell barrier function by inducing persistent activation of eNOS and increasing the activity of the downstream NO target, soluble guanylate cyclase via $S1P_1$. Kimura et al. (67) have demonstrated that HDL-S1P modulates endothelial cell proliferation and survival by activating extracellular signal-regulated kinase (ERK) and that endothelial migration was sensitive to both $S1P_1$ and $S1P_3$, while cell survival was exclusively sensitive to $S1P_1$ (68). The same group later showed that HDL inhibits the production of MCP-1 in endothelial cells and this process is mediated by the coordinate signaling through $S1P_3$ and SR-BI receptors. The expression of many adhesion molecules is regulated by ROS-dependent mechanisms. HDL-S1P attenuates ROS production by inducing NO production and inhibiting NADPH oxidase (69). Tölle M et al. (12) reported a similar HDL-S1P induced MCP-1 and NADPH oxidase inhibitory effect in VSMCs via $S1P_3$. HDL also induces the endothelial expression of long pentraxin 3 (PTX3) (70) and transforming growth factor β (TGFβ) (71), both of which are key modulators of immune response. The authors attributed these observed effects to the activation of $S1P_1$ and $S1P_3$ by HDL-S1P. Interestingly, HDL appears to have carrier-specific effects on S1P signaling. Compared with albumin bound S1P, HDL-S1P induces prolonged S1P-$S1P_1$ signaling involving persistent activation of Akt and eNOS. HDL as a carrier, reduces the rate of $S1P_1$ protein degradation and promotes recycling of internalized $S1P_1$ (66). In addition, HDL receptor systems, such as SR-BI, may cooperate with S1P receptor systems in HDL induced

signaling (69) and part of the effects may be attributed to the particle docking function of HDL receptors on the cell surface (16).

As the protective role of HDL-S1P has been convincingly illustrated in molecular and cellular studies, in recent years, researchers have started to conduct human studies and examine the status of HDL-S1P in disease conditions. In two separate studies, HDL-S1P have been reported to be lower in patients with stable coronary artery disease (CAD) than in healthy controls and that HDL-S1P is inversely associated with the occurrence of coronary artery disease, independent of HDL-C concentration (72, 73). Further research has showed that HDL-S1P is inversely associated with the severity of CAD and is an independent predictor for coronary in-stent restenosis (74, 75).

Oxylipins

Oxylipins are a structurally and functionally diverse array of lipid mediators derived from polyunsaturated fatty acids (PUFA). Over the last decade, oxylipins have emerged as important regulators of inflammatory progress and have been implicated in various inflammatory conditions including type 2 diabetes, cardiac surgery, proteinuric hyperlipidemia and Immunoglobulin A nephropathy (76-79). They can be generated either via auto-oxidation by ROS or via the actions of three enzyme families including COX, LOX, and cytochrome P450 (CYP)(80). PUFAs are susceptible to auto-oxidation and the main products include hydroperoxides, hydroxyl-FA and prostanoids (81). The COX pathway leads to the generation of the pro-inflammatory prostaglandin H2 (PGH2), PGF2a, thromboxane A2 (TXA2) and TXB2 as well as the anti-inflammatory prostaglandin E2 (PGE2), PGD2, and prostacyclin (PGI2). The LOX pathway contains

three types of enzymes 5-LOX, 12-LOX and 15-LOX. The 5-LOX pathway generates a series of pro-inflammatory lipid mediators from arachidonic acid (AA) including 5-hydroxyeicosatetraenoic acids (5-HETE), 5-keto-eicosatetraenoic acid (5-KETE) and leukotriene B4 (LTB4) that promote leucocyte chemotaxis (82). n-3 PUFA compete with AA for 5-LOX pathway to form less potent metabolites and contributes to the reduction of inflammation (83). On the other hand, the products of 12-/15-LOX pathway can have dual pro-inflammatory and pro-resolution activities (84). The CYP pathway contains two types of enzymes: CYP hydroxylase and CYP epoxygenase, which give rise to alcohol and epoxide metabolites respectively. Epoxides contribute to the maintenance of vascular homeostasis by promoting vasodilation and angiogenesis and suppressing inflammation (85). Epoxides can be further metabolized by soluble epoxide hydrolase (sEH), yielding diols. Degradation of epoxides causes them to lose their athero-protective efficacy. Cellular oxylipins are mainly attached to phospholipids on cell membranes and their enzymatic generation depends on cell type (86). Also, the metabolites of the different pathways may not be unique to that specific pathway. For instance, the 15-LOX product of AA 15-HETE can also be generated from COX and CYP pathways as well as by auto-oxidation (87-89).

The best-characterized oxylipins are AA derived prostanoids including prostaglandins and thromboxanes via the COX pathway; they are not known to become esterified into phospholipids. In contrast, CYP and LOX derived metabolites can become incorporated into cellular phospholipids as well as neutral triglycerides and cholesterol esters. Over 90% of circulating oxylipins occur as esterified forms in lipoproteins (90)

and they are differentially distributed among different lipoprotein compartments. For example, 13-hydroxyocta-decadienoate (13-HODE), the linoleic acid (LA) derived alcohol product, which has been reported to promote macrophage accumulation in plaques (91), is most enriched in HDL. In fact, HDL particles contain the highest load of alcohols, epoxides and total oxylipins (90). So far, little is known about the sources for lipoprotein oxylipins and how they are metabolized. But HDL particles are relatively small and can readily enter extracellular spaces. HDL also lacks heparin binding site and thus is normally not retained in extracellular matrices. The unique physiochemical property of HDL particles makes them an ideal shuttle platform for lipid transportation. This property may account for their distinctive oxylipin profile. As enzymatically derived oxylipins are exclusively generated inside cells and incorporated into cell surface, it is possible that HDL acquires the oxylipins from peripheral cells and immune cells such as macrophages and neutrophils as well as other lipoprotein particles in the same manner they acquire triglycerides and phospholipids. It is also unclear whether HDL oxylipins are mainly targeted for hepatic disposal or if they function in an endocrine manner. It has been demonstrated that esterified oxylipins in VLDL can be liberated by lipoprotein lipase and act as lipid mediators (78). Alternatively, 5-LOX derived oxylipins esterified in phospholipids can modulate immune response in neutrophils and it would be interesting to know whether HDL oxylipins directly interact with cell surface oxylipin receptors and whether HDL receptors such as SR-B1 and ABCA1 participate in a co-ordinated signaling with oxylipin receptors.

With the improvement in the accuracy, sensitivity and speed of liquid chromatography and mass spectrometry as well as the development of bioinformatics (77, 92), oxylipin profiles can now be generated rapidly and be used to discriminate disease phenotypes. A handful of studies demonstrated the alteration in circulating oxylipin profile during inflammation and oxidative stress, both of which are well-defined atherothclerosis risk factors. In type 2 diabetes patients, plasma epoxides and ketones of 18 carbon PUFA were increased by over 80% and were positively associated with changes in plasma non-esterified fatty acids (NEFA) (93). During cardiac surgery, the activity of LOX pathway was up-regulated and elevated concentrations of the chemoattractants 5-HETE and 12-HETE were observed (77). As circulating oxylipins are differentially distributed, it remains to investigate how oxylipin profiles of different lipoprotein subclasses compare to total plasma oxylipin profile in disease risks prediction.

HDL remodeling and functionally defective HDL

While HDLs are generally perceived as athero-protective particles and HDL-C has been identified as an independent predictor for ASCVD, a significant number of coronary heart disease (CHD) events occur in individuals with normal HDL-C. Indeed, in the original Framingham study, over 40% (44% for men and 43% for women) of the CHD events occur in men with HDL-C ≥40 mg/dL and women with HDL-C ≥50 mg/dL (94). Consequently, continuing efforts have been made in study of HDL functions and in search of markers with better predictive value on the individual level. The concept of dysfunctional HDL was first developed about 20 years ago when Fogelman and

colleagues (95) found that the normally anti-inflammatory HDL was converted to a pro-inflammatory particle during an acute phase response in both humans and a croton oil rabbit model. They constructed an "inflammatory index" for HDL, as defined by the relative capacity of HDL to inhibit monocyte chemotaxis induced by oxidized LDL. In a seminal study, this group demonstrated that HDL from patients with CHD who had normal concentrations of HDL-C, had reduced anti-inflammatory activity when compared with healthy controls (96). In fact, the HDL from many CHD patients had increased monocyte chemotaxis in response to oxidized LDL. A number of other studies further showed that HDL can also display a complete loss of vasodilatory (97, 98) and antiapoptotic function (99), as well as a partial loss of antioxidative (100, 101) and cholesterol efflux function (102-104). Chronic inflammation is characterized by increased oxidative stress and overproduction of inflammatory protein by the liver and macrophage, which triggers pronounced protein and lipid remodeling of HDL with the subsequent disruption of HDL athero-protective functions.

Oxidative change

In response to pro-inflammatory cytokines and growth factors, several phospholipases, in particular cytosolic phospholipaseA2 (cPLA2), are activated and release PUFA from cell membranes (105). Free PUFA can undergo peroxidation via enzymatic and non-enzymatic pathways and forms the intermediate hydroperoxides, which lead to the generation of the alcohol and ketone products. Excess generation of ROS and activation of LOX enzymes during inflammation contribute to enhanced production of oxidized lipids. In fact, 5-LOX and cPLA2 share the same structural (C2

domain) and regulatory (activation by Ca^{2+} and by phosphorylation) properties and are often co-regulated (106). In chronic inflammatory mice models, HDL oxylipin profiles were shown to possess increased 5-HETE and 9-HETE, which are biomarkers for 5-LOX activation and auto-oxidation respectively (78). Human studies have shown that HDL from patients with diabetes and CVD are more enriched with LA (9-HODE, 13-HODE) and AA (5-HETE, 12-HETE, 15-HETE) derived alcohol oxylipins compared with healthy controls (107, 108), despite a similar CVD risk factor profile (similar age, high blood pressure, lipid profile, and overweight). The authors speculated that the accumulation of oxidized lipids play a role in the observed PON1 inactivation and HDL dysfunction. In addition to lipids, HDL proteins can also be oxidized during inflammation. Myeloperoxidase (109) and 15-LOX (110) are two oxidative enzymes capable of ApoA-I modification and have been implicated in human atherosclerotic lesions. Leukocyte released myeloperoxidase mediates carbamylation of lysine residues and oxidation of methionine and tryptophan residues on ApoA-I and thereby diminishes ABCA1 dependent cholesterol efflux and LCAT activity (111-116) as well as eNOS expression and activity (117). 15-LOX can induce ApoA-I and ApoA-II cross-linking and thereby decreases SR-BI dependent cholesterol efflux from macrophages (118).

Protein/lipid distribution change

Serum amyloid A (SAA) is a family of apolipoproteins overproduced during acute phase response that are capable of displacing ApoA-I in HDL (119). HDL proteomics analysis showed that ApoE preferentially resides in the large HDL_2 subfraction. A shift in the distribution of the majority of ApoE towards smaller HDL

particles has been reported in CAD and diabetic patients (33, 120). It has been hypothesized a shift of ApoE from HDL_2 to HDL_3 may impair ApoE mediated cholesterol efflux from macrophages. HDL-S1P has been found to be lower, whereas non-HDL-S1P were higher in patients with myocardial infarction and CAD, when compared with healthy individuals, suggesting that the health status of an individual may affect the partitioning of S1P to HDL (72-74). As mentioned above, HDL has carrier specific effects on S1P bioactivity and the shift of S1P towards other lipoproteins or albumin may impair HDL functionality. In support of this assumption, Sattler et al. (72) reported that the concentration of HDL-S1P is negatively associated with the severity of CAD symptoms, whereas that of non-HDL-S1P increases proportionally with the severity of CAD. The same group recently demonstrated that the reduction of HDL-S1P in CAD patients translates to impaired HDL signaling. The reduction is possibly caused by oxidative modification of HDL constituents such as ApoM. S1P enrichment in CAD-HDL by pharmacological blockade of S1P lyase or by administration of S1P-loaded erythrocytes corrects HDL dysfunction. It should be noted that HDL is highly heterogenous in composition. HDL_3 is preferentially enriched in the anti-oxidant enzyme PONase and PAFAH (120), the anti-inflammatory phospholipid S1P and its carrier ApoM (121), as well as pro-inflammatory proteins such as SAA1 and SAA2 (120). While the HDL_3 subclass has higher arthero-protective capability under normal conditions (44), it is also more susceptible to compositional change and function loss during inflammation.

Density distribution change

HDL proteins and lipids play key roles in HDL metabolism, thus a change in composition is often associated with altered metabolism. ApoA-I oxidation and displacement impairs ABCA-I dependent cholesterol efflux. In the presence of myeloperoxidase metabolites such as hypochlorous acid (HOCl) or hydrogen peroxide, HDL_3 become unable to activate LCAT (116). Secretory phospholipaseA2 ($sPLA_2$), a potent inflammatory reactant, can hydrolyze HDL phospholipids, reducing surface fluidity of the particle, and therefore impair SR-BI and ABCG1 dependent cholesterol efflux (25, 122). Further, cholesterol transporters including ABCA1, ABCG1 and SR-BI have all been reported to be down-regulated during inflammation (123, 124). The diminished RCT capacity prevents small HDL from maturation and often cause a shift in HDL density distribution. Indeed, small spherical HDL phenotype has been reported to be associated with increased risk for CHD and acute ischemic stroke independent of HDL-C (125-127). CVD patients with normal HDL-C may result from decreased HDL-C turnover through hepatic uptake and utilization instead of fully functional RCT. Other inflammatory disorders such as metabolic syndrome, type 2 diabetes and obesity are also characterized by the disappearance of large buoyant HDL and the prevalence of small dense HDL (128-130), though the increased small dense HDL may be partially attributed to the intravascular lipolysis of triglyceride-enriched HDL.

To sum up, the process of HDL-C metabolism is complex and the functionality of HDL is largely dependent on its composition, which can alter in a variety of disease states. Steady-state levels of HDL-C provides little information on the actual rate of

cholesterol efflux, nor on HDL's anti-inflammatory, anti-oxidant and endothelial protecting activities. Therefore, it has been proposed that HDL subclass distribution and composition may be better determinant of HDL mediated athero-protection and their measurement may be superior to HDL-C in predicting cardiovascular disease (33, 131, 132).

Tools for HDL subclass measurement

HDL is a heterogeneous mixture of particles, which are composed of a hydrophobic lipid core and an outer shell of amphipathic lipids and proteins with distinct chemical and physical properties. Based on density, size, surface charge, lipid methyl groups and protein composition, lipoprotein subclasses can be measured by various analytic methods including ultracentrifugation, gel electrophoresis, precipitation-based methods, nuclear magnetic resonance (NMR) spectroscopy, ion mobility etc. The use of different techniques and procedures has led to different terms in defining HDL species. Ultracentrifugation is a classic method used for HDL separation and subclass identification on the basis of hydrated density. Sequential ultracentrifugation divides spherical HDL particles into two main subclasses: large, light, lipid rich HDL_2 (d = 1.063-1.125 g/mL) and small, dense, protein rich HDL_3 (d = 1.125-1.21 g/mL). With density gradient ultracentrifugation, HDL can be further divided into 5 subclasses in order of decreasing diameter and increasing density, HDL_{2b} (d = 1.063-1.091 g/mL), HDL_{2a} (d = 1.091-1.110 g/mL), HDL_{3a} (d = 1.110-1.133 g/mL), HDL_{3b} (d = 1.133-1.156 g/mL) and HDL_{3c} (d = 1.156-1.179 g/mL). The density intervals of the 5 subclasses are defined to match up with the 5 subclasses that are identified using polyacrylamide

gradient gel electrophoresis: HDL_{2b} (9.7-12.9 nm), HDL_{2a} (8.8-9.7 nm), HDL_{3a} (8.2-8.8 nm), HDL_{3b} (7.8-8.2 nm), HDL_{3c} (7.2-7.8 nm).

The earliest HDL isolation method is analytical ultracentrifugation, which was developed over 70 years ago. HDL subclasses are separated by flotation rate in a salt solution (neutral salts, such as NaBr or KBr) of density 1.2 g/mL and their total mass are quantified by Schlieren optics. Three HDL subclasses, i.e., HDL_1, HDL_2 and HDL_3, can be separated. HDL_1, the subclass with the highest flotation rate, is generally not detectable in human plasma (133). In the early 1980s, an isopycnic, density gradient ultracentrifugation approach was developed by Chapman et al. (134) which can separate HDL into 5 subclasses (HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3b}, HDL_{3c}) in a single spin. This method requires 3 mL plasma or serum and blood samples are centrifuged in a swing-out rotor at 40k rpm (288,000*g* at r_{max}) for 48 h. The density gradient is constructed by consecutive layering of 4 NaCl-KBr solutions of distinct densities at centrifugation temperature. Vertical auto profile (VAP) is another single spin, density gradient based ultracentrifugation technique (135). With the use of a vertical rotor (80k rpm, 509,644*g* at r_{max}) and two pre-formed density gradient layers (NaCl and NaCl-KBr), all lipoproteins can be separated in less than 1 h. Following ultracentrifugation, VAP measures cholesterol content in two major HDL subclass, HDL_2 and HDL_3, by a controlled-dispersion flow analyzer. This method is sensitive, requiring only 50 µL plasma or serum. However, as lipoproteins are resolved along the long axis of the tube during ultracentrifugation, they will reorient to the horizontal axis upon completion of ultracentrifugation. The high degree of reorientation may result in contamination of

lipoprotein classes. Analytic and preparative ultracentrifugation are gold standards for lipoprotein subclass separation as they are precise and reproducible (136). However, these ultracentrifugation methods have a few common limitations. Exposure to high ionic strength and high centrifugal force can result in apolipoprotein loss in HDL particles (137). Moreover, they are either labor intensive (manually prepare pre-formed density gradient) or time consuming.

More recently, a novel and rapid method for lipoprotein density profiling has been reported using the combination of metal ion EDTA density gradient ultracentrifugation, fluorescent staining of lipoproteins and digital imaging (138, 139). Pre-stained lipoproteins are separated by metal ion EDTA density gradient ultracentrifugation and quantified by measuring the distribution of fluorescent dye. With the use of a high speed, fixed angle rotor (120k rpm, 627,000*g* at r_{max}), a lipoprotein density profile can be achieved within 6 h. Metal ion EDTA salts as density gradient medium have the advantage of high molecular weight, small partial volume, and low viscosity, allowing rapid formation of a gradient from a homogeneous solution as well as reduced ionic strength and accumulated centrifugal force. The elimination of the need for step gradients and adjustments for density solution by salt addition also provides greater reproducibility than conventional ultracentrifugation methods. Further, fluorescence imaging is a highly sensitive quantification method, requiring only 6 µL plasma or serum for a high resolution profile. As lipoprotein subclass are identified by their hydrated density, this approach requires density gradient formation characterization for individual rotors to insure accuracy.

Dietary fatty acids and ASCVD risk

The quantity and quality of dietary fats are major regulators of blood lipid profile and ASCVD risk. SFA is long known to elevate LDL-C, a primary risk factor for ASCVD (140). In addition， SFA can increase ASCVD risk by promoting the production of inflammatory cytokines, impairing endothelial function and insulin sensitivity (141-143). Reduction in dietary SFA intake (<7% total energy) is recommended by the American Heart Association and National Cholesterol Education Program for ASCVD prevention and treatment (144, 145). For energy balance purpose, the reduction in SFA intake requires its replacement by other macronutrients. The beneficial effects of high MUFA diets initially received attention as multivariate analysis showed that MUFA enriched Mediterranean diets were associated with decreased incidence of CHD (146). Compared with a high SFA average American diet (34% fat; 16% SFA, 11% MUFA) diet, high MUFA diets (34% fat; 7% SFA, 18-21% MUFA) lowers LDL-C by similar magnitude as a high carbohydrate, low fat NCEP Step II diet (25% fat; 7% SFA, 12% MUFA) in healthy normocholesterolemic individuals. However, such diets did not elevate TAG or decrease HDL-C concentration, as did a high carbohydrate, low fat diet (147). Meta-analysis of 60 controlled trials showed that when carbohydrates constituting 1% of dietary energy were replaced isoenergetically with MUFA, total-C:HDL-C and ApoB were reduced. No such change was observed when carbohydrate was substituted by SFA (148). In subjects at risk of metabolic syndrome, a high SFA diet (19% SFA, 11% MUFA) has been reported to up-regulate the expression of inflammatory genes in adipose tissue, while a high MUFA olive oil diet

(11% SFA, 20% MUFA) promoted the expression of anti-inflammatory genes (149). Further, isoenergetic substitution of SFA by MUFA have been shown to decrease diastolic blood pressure, improve flow-mediated dilation (endothelial function assessment) and decrease concentration of the cell adhension molecule P-selectin in healthy subjects, all of which changes were associated with reduced ASCVD risk (142, 150).

Beef consumption and ASCVD risk

Beef is the most commonly consumed red meat in the US. The U.S. Department of Agriculture's (USDA) 1994-1996 Continuing Survey of Food Intakes by Individuals (CSFII) showed that approximately 75% of the population including adults and children consumed beef during a 2-day period (151). Beef is a naturally nutrient-rich food providing high amounts of protein, vitamin B6 and minerals such as iron and zinc (152). Beef is also a major contributor to MUFA intake in US (153, 154). Oleic acid is the most abundant MUFA in beef and is converted from stearic acid by Δ9 desaturase (155). The fatty acid profile of beef can be modulated by altering the diets of cattle. It has been reported that activity of stearoyl-CoA desaturase (SCD) gene, which encodes Δ9 desaturase, is higher in corn-fed steers than pasture-fed steers (156) and contributes to the higher MUFA content in corn-fed beef (157, 158). Beef consumption pattern analysis indicates that ground beef held the highest market share with about 42% (159), and over 65% of the ground beef consumed in the US contains 16-30% fat. In retail outlets, ground beef MUFA:SFA ratio varies widely and can range from 0.84 to 1.46 (160).

While some epidemiological studies reported adverse health associations for red meat, this may be attributed to the high SFA intake (161). As MUFA has been suggested to be more beneficial than SFA and that MUFA:SFA has a wide range in beef, the effect of beef fatty acid composition on ASCVD risk factors has been evaluated by several studies. In a dietary intervention study, 10 mildly hyper-cholesterolemic free-living men consumed two types of ground beef patties (114 g, 35%wt fat) of differing MUFA:SFA ratios for 5 wk with a 3 wk washout period between the diets (160). During the feeding periods, the participants were first on a low MUFA (M:S = 0.95, pasture-fed) beef diet and then rotated to a high MUFA (M:S = 1.31, corn-fed) beef diet. Results showed that the low MUFA beef diet increased plasma concentration of TAG and reduced concentration of HDL-C. And these adverse effects on lipid profile were abrogated after consumption of the high MUFA:SFA beef diet, indicating the high MUFA beef either reversed the negative effects of the low MUFA beef diet or at least was neutral in its effects on plasma lipids. In a subsequent study, the health effects of beef fatty acid composition were evaluated in 27 healthy free-living men in a random crossover design (162). The two beef patties designed for the study were 114 g, 24%wt total fat, with a M:S = 0.71 for low MUFA beef and a M:S = 1.1 for high MUFA beef. The participants completed two 5 wk feeding trials in which 5 patties were consumed per week and the initial assignment to low or high MUFA beef was random. Results showed that HDL-C had a trend to increase with the consumption of both beef diets. However, the elevation was only significant for the high MUFA beef diet. It remains unknown whether the increase in HDL-C translates to an increase in functional HDL particles.

On the other hand, the SFA intake recommended by the American Heart Association was <7% of energy. Dietary Approaches to Stop Hypertension (DASH) is a gold standard heart healthy diet which features more fruit and vegetable as well as less SFA, cholesterol and red meat intake (163, 164). To test whether lean beef could be included in an athero-protective diet, the Beef in an Optimal Lean Diet (BOLD) study compared the cholesterol lowering effects of DASH and two different low SFA, lean beef enriched DASH-like diets in a mildly hypercholesterolemic population (165). A primary outcome was that lean beef as main protein and partial replacement of carbohydrates by protein (including lean beef) in DASH-like diet decreased LDL-C, HDL-C and total-C by a similar magnitude as DASH diet. It remains unknown the effect of beef as a main protein source in a DASH-like diet on lipoprotein density distribution and how that may be influenced by total fat intake.

Present study

Objectives:

1. To determine the effects of ground beef fatty acid composition on HDL density distribution, HDL oxylipin profile and HDL_3 composition in healthy men consuming ground beef containing 24% fat of differing degrees of saturation.
2. To validate the method of lipoprotein density profiling by NaBiEDTA density gradient ultracentrifugation using a fixed angle MLA-130 rotor and apply the method to HDL density distribution analysis.
3. To compare the effects of beef consumption on HDL density profiles in two separate populations with differing total fat intakes as an add-on study.

CHAPTER II

GROUND BEEF ACID COMPOSITION INFLUENCES HDL_3 SPHINGOSINE-1-PHOSPHATE CONTENT AND IS RELATED TO ATHEROSCLEROTIC CARDIOVASCULAR DISEASE RISK FACTORS IN HEALTHY MEN

Introduction

Dyslipidemia, glucose intolerance and oxidative stress are strongly associated with increased ASCVD risk (2). Low HDL-C is a common dyslipidemia identified by the National Cholesterol Education Program Adult Treatment Panel III as an independent risk factor for ASCVD (2, 166). However, large clinical trials to increase HDL-C through pharmacologic agents show no clear ASCVD risk reduction with increases in HDL-C concentration (166). HDL particles are highly heterogeneous in physiochemical and biological properties (166). Accumulating evidence suggests that small HDL_3 particles can lose their functionality for RCT, anti-inflammation and anti-oxidant activity or even become atherogenic under inflammatory states due to compositional changes in lipid and protein components (131, 167). Furthermore, ASCVD patients with higher concentrations of HDL_3 in the presence of increased larger VLDL were demonstrated to be 3-4 times more likely to develop extensive coronary artery disease (168). In diabetic patients, amounts of HDL_3 tend to comprise a greater proportion of total HDL mass independent of serum triglycerides (TG) and total HDL-C (169).

Increases in HDL-C with higher intakes of SFA and MUFA is well documented (170). However, whether such dietary induced increases result in increased amounts of functional HDL is not known. In our previous study, subjects consuming 5, 114g ground-beef patties (27 g fat/114g patty of either high (1.10) or low (0.71) MUFA:SFA) per week for 5 wk exhibited increased HDL-C, with a greater (P <0.05) increase observed following high MUFA ground beef consumption (162). Average diameters of HDL_2 and HDL_3 were similar across groups but the proportion of HDL in each diameter subfraction was not determined. This determination was of interest as the small and dense HDL_3 subclass is believed to include both the most beneficial and the most detrimental species of HDL (171, 172). Thus changes in the amount of this HDL subclass can have different biological actions. Ambiguity in risk/benefit assignments for HDL subclasses currently limits the diagnostic value of traditional measures such as total HDL-C.

In the present study, the effects of ground beef consumption on HDL density distribution, HDL_3 apolipoprotein composition and S1P content were measured. We also examined whether the fatty acid composition of ground beef influenced its effects on HDL properties as well as metabolic indices of insulin sensitivity, oxidative stress markers and adipokines.

Methods

Subjects and design

Complete sets of banked plasma and serum samples were analyzed from 25 healthy men (Table 2.1) who successfully completed a beef feeding trial (162). Briefly,

participants consumed ground beef with a total fat content of 24%, but in which the MUFA:SFA was either high (1.10:1) or low (0.71:1). All subjects consumed both types of ground beef, with random initial assignment to the low MUFA or high MUFA treatment. All subjects were free-living, but were instructed to replace dietary protein of one meal per day with one 114 g ground beef patty for 5 d/wk. Each dietary phase lasted 5 wk and was followed by a 4 wk wash out period prior to starting the next dietary phase. Blood samples were collected following a 14 h fast at the start and end of each feeding period. Separated plasma and serum samples were stored at -80 °C prior to analysis.

Table 2.1 Baseline characteristics of Patty Study subjects

Variables	
Age (y)	35.4 ± 2.2
BMI	27.1 ± 0.8
TG (mg/dL)	105.0 ± 8.8
TC (mg/dL)	187.0 ± 7.3
LDL-C (mg/dL)	120.8 ± 6.6
HDL-C (mg/dL)	45.6 ± 1.7

Data expressed as mean ± SEM. n = 25.

Laboratory measurement

Serum glucose was measured using an enzymatic assay kit (Cat. #GAHK20, Sigma-Aldrich, St. Louis, MO) and the activity of PON1 was measured by using freshly prepared phenylacetate (Sigma-Aldrich, St. Louis, MO) as substrate and determined by spectrophotometric assay (173). Serum insulin was assayed using an ELISA kit (Cat. #EZHI-14K, Millipore, Billerica, MA). Homeostatic model assessment (HOMA) score was calculated from serum glucose and insulin concentration using a HOMA2 calculator

(174) as an estimate of insulin sensitivity. HDL_3 (d = 1.125-1.21 kg/L) was isolated from plasma by NaCl-NaBr sequential flotation ultracentrifugation and dialyzed against 0.9% saline overnight (175). Serum adiponectin, resistin and plasminogen activator inhibitor 1 (PAI-1) and HDL_3 ApoA-I, ApoA-II and ApoE were measured by multiplex immunoassay kits (Cat. #HADK1-61K-A and Cat. #APO-62K, Millipore, Billerica, MA) using a Luminex analyer (Luminex, Austin, TX).

Serum homocysteine (S-Hcy) and HDL_3 S1P were analyzed by HPLC as previously described with modifications (16, 176). Plasma HDL_3 S1P was measured since over half of the plasma S1P is carried by HDL and most concentrated in the HDL_3 subfractions (121, 177). Protein bound homocysteine was converted to the free thiol form by incubation with 100 µL of 1% 2-mercaptoethanol (β-ME) in 40 mmol/L sodium borate solution, pH 9.5 at 37 ºC for 30 min. The remaining protein was acid precipitated using 100 µL of 1.5 mol/L $HClO_4$, and then neutralized with 50 µL of 2 mol/L K_2CO_3 prior to addition of 200 µL 30% (w/v) Brij® 35 solution and 50 µL of an iodoacetic acid solution (52 mg iodoacetate, 5 mL 40 mmol/L sodium borate, pH = 9.5). After derivatization with 200 µL ortho-phthalaldehyde (OPA) solution (25 mg OPA, 625 µL methanol, 25 µL β-ME, 6.25 mL 0.04 mol/L sodium borate, pH = 9.5), homocysteine was measured using a Dionex P580 HPLC system (Dionex, Sunnyvale, CA) equipped with a 5 µm, 150 × 4.6 mm SUPELCOSIL™ LC-18 column (Sigma-Aldrich, St. Louis MO) and Dionex RF2000 fluorescent detector (Dionex, Sunnyvale, CA) set at 340 nm excitation and 450 nm emission wavelengths. The mobile phase was 86:14 solution A (0.02 mol/L NaAc, 2.68 mmol/L triethanolamine, 0.4%v tetrahydrofuran, pH = 7.2):

solution B (10 mmol/L NaAc/methanol/methyl cyanide 5:1:1 v/v/v, pH = 7.2) at 0.8 mL/min. Concentration of S1P in HDL_3 were measured following addition of 20 µL of 80 ng C17-S1P /mL internal standard to 100 µL of isolated HDL_3. Selective extraction of S1P was accomplished by liquid/liquid extraction with 1 mL methanol containing 2.5 µL concentrated HCl, 1 mL chloroform, 200 µL 4 mol/L NaCl and 100 µL 3 mol/L NaOH. The alkaline aqueous phase was then transferred to a clean tube and the organic phase was re-extracted with 0.5 mL methanol, 0.5 mL 1 mol/L NaCl and 50 µL 3 mol/L NaOH. The aqueous phases were combined, acidified with 100 µL concentrated HCl and extracted twice with 1 mL chloroform. The S1P-containing chloroform was dried at 50ºC under nitrogen. For HPLC analysis, S1P was redissolved in 100 µL mobile phase (methanol, 70 mmol/L K_2HPO_4, 78:22 v/v) and combined with 100 µL OPA solution (25 mg OPA, 625 µL methanol, 25 µL β-ME, 6.25 mL 40 mmol/L sodium borate, pH = 10.5). The OPA derivatives of S1P were analyzed at a flow rate of 0.25 mL/min using a 5 µm, 150 x 2.1 mm Kromasil C18 column (Sigma-Aldrich, St. Louis MO) and detected by a Dionex RF2000 detector (Dionex, Sunnyvale, CA) using an excitation wavelength of 340 nm and an emission wavelength of 456 nm. A calibration curve was generated using serial dilutions of S1P standards. S1P was quantified by comparison of its fluorescent intensity with that of the internal standards.

Density profiles for circulating lipoproteins were determined by imaging 6 µL serum following 6-((N-(7-Nitrobenz-2-Oxa-1,3-Diazol-4-yl)amino)hexanoyl) sphingosine (NBD-C6-ceramide) labeling of lipoproteins exactly as described except for a change in the respective filters (Semrock, Rochester, NY), namely 465 nm/60 nm

bandwidth, part #FF01-460-60-25 for excitation and >500 nm/long pass, part # BLP01-488R-25 for emission (178). Overall lipoprotein density profile was analyzed as absolute area under the curve where image area was measured as pixels (AUC, i.e. number of pixels within a density interval) and data were also used to express HDL subfractions as percentages of total HDL AUC. The average percent relative standard deviations (%RSD) in AUC for different lipoprotein subfractions were 4.45% (within-day) and 7.37% (day-to-day). To test the effects of sample storage on density distributions, serum samples from six non-study subjects were divided into multiple aliquots in order to evaluate differences in density distributions of never frozen samples to those of samples that had been thawed once, twice, or three times. To test the protein binding properties of NBD-C6-ceramide, 6 µL 50 g bovine serum albumin (BSA)/L saline and apolipoprotein calibrator containing 660 mg ApoA-I, 290 mg ApoA-II and 360 mg ApoE/L were used (Millipore, Billerica, MA). The protein solutions were prepared and analyzed by the same methods as serum samples.

Statistics

Data analysis was performed using JMP 10.0 (SAS Institute Inc, Cary, NC). The distributions of variables were assessed by Shapiro-Wilk test and log transformations of skewed variables were used in subsequent analyses. The effect of beef consumption on ASCVD risk factors was analyzed by paired t-tests against relevant baseline values. Baseline values were averaged for tabular presentation. Simple correlations were tested by Pearson's correlation coefficient. Cook’s D distance was calculated and the cutoff point for outliers was 4/n. A one-way analysis of covariance was used to adjust lipid

confounders for the correlation between HOMA-IR and HDL density distribution. Data were expressed as mean ± SEM. The level of significance was P <0.05.

Table 2.2 Effect of low or high MUFA beef intervention on serum ASCVD risk factors, HDL density distribution and HDL_3 composition

Variable	Baseline	Low MUFA	High MUFA
Serum ASCVD risk factors			
HOMA-IR	0.84 ± 0.11[a]	0.69 ± 0.12[b]	0.66 ± 0.12[b]
S-Hcy (μmol/L)	11.6 ± 0.4	12.5 ± 0.6	12.3 ± 0.5
PON1 (kU/L)	57.3 ± 1.5	54.7 ± 2.3	56.7 ± 2.2
PAI-1 (μg/L)	18.8 ± 1.2	19.2 ± 1.7	18.6 ± 1.3
Resistin (μg/L)	31.6 ± 1.9	29.7 ± 2.4	30.2 ± 2.3
Adiponectin (mg/L)	15.0 ± 1.5	15.1 ± 1.5	14.1 ± 1.3
Total lipoprotein, pixels	4620 ± 131	4591 ± 136	4599 ± 118
Total TRL, pixels	330 ± 35	309 ± 43	290 ± 38
Total LDL, pixels	2109 ± 94	2025 ± 95	2087 ± 110
Total HDL, pixels	2181 ± 58[b]	2257 ± 63[a]	2222 ± 60[a,b]
HDL_2/HDL_3	1.01 ± 0.07	1.11 ± 0.06	1.05 ± 0.06
HDL density distribution (% of total HDL AUC)			
HDL_{2b}	20.2 ± 0.9	20.7 ± 1.0	20.1 ± 1.0
HDL_{2a}	20.4 ± 0.5	21.0 ± 0.4	20.5 ± 0.5
HDL_{3a}	27.4 ± 0.5	27.2 ± 0.5	27.3 ± 0.5
HDL_{3b}	19.9 ± 0.6	19.2 ± 0.6	19.9 ± 0.6
HDL_{3c}	12.2 ± 0.4	12.0 ± 0.4	12.2 ± 0.3
HDL_3 composition			
ApoA-I (mg/L)	439.2 ± 19.2	415.2 ± 17.8	436.8 ± 20.9
ApoA-II (mg/L)	77.6 ± 6.2	78.6 ± 7.1	84.1 ± 10.1
ApoE (mg/L)	3.22 ± 0.38	3.15 ± 0.37	3.56 ± 0.40

All data were expressed as mean ± SEM. n = 25. Two baseline values were averaged for table. Effect of beef intervention was determined by paired t-test. Values with different superscript letters were significantly different pairwise (P <0.05). Lipoprotein density distribution images were measured as pixels. Total pixels were set equivalent to total area under the curve (AUC).

Results

The baseline characteristics and dietary intake information for test subjects have been previously published (162). Briefly, each type of beef patty provided 27 g of fat. The low MUFA patty provided 15 g SFA, 11 g MUFA, 1.3 g trans-fat and 0.85 g 16:1-n7. Each high MUFA patty provided 13.9 g SFA, 14.5 g MUFA, 0.92 g trans-fat and 0.97 g 16:1-n7. Compared to baseline intakes, 5 wk of either low MUFA or high MUFA beef consumption decreased HOMA-IR score similarly, 11.1% ($P = 0.03$) and 10.1% ($P = 0.03$), respectively (Table 2.2). Here data were expressed as mean of individual percentage changes. There was one particular negative responder whose HOMA-IR increased 155% and 212% after the two beef diets, the exclusion of this subject would make the decrease in HOMA-IR 17.7% and 19.7% for low MUFA and high MUFA diets respectively. There was no change from baseline in the activity of PON1, concentrations of homocysteine or PAI-1, adiponectin or resistin after either beef intervention (Table 2.2).

Lipoprotein density distribution profile stability

Figure 2.1 shows the lipoprotein density profile for a representative responder. HDL_{3a} was the most abundant HDL subfraction, followed by HDL_{2a}, HDL_{2b} and HDL_{3b}, with HDL_{3c} being the least abundant subfraction. The distribution of the NBD-ceramide stained apolipoprotein standards and BSA solution following NaBiEDTA density gradient ultracentrifugation is shown in overlay (Figure 2.2). The apolipoprotein standard shows three small, closely spaced, peaks within the d >1.20 kg/L region of the density gradient. The BSA solution showed a tall peak in this same region which

corresponded to a similarly large peak in the native serum sample, presumably human albumin. Our stability study showed that there was no significant difference in total HDL AUC or HDL density distribution following as many as three freeze-thaw cycles (Table 2.3).

HDL density distribution and HDL_3 composition

Compared to baseline distributions, low MUFA beef increased total HDL AUC by 3.5% ($P = 0.03$) while no effect was observed for high MUFA beef (Table 2.2). Neither beef intervention altered the fractional abundance of the 5 major HDL subclasses measured. Similarly, ApoA-I, ApoA-II and ApoE concentrations in HDL_3 were unaffected by beef consumption. However, HDL_3-S1P concentration (µg/mg ApoA-I) was increased by 15.6% ($P = 0.05$) by high MUFA beef consumption relative to baseline diet concentration (Figure 2.3). A diminished 7.5% increase over baseline HDL_3-S1P was observed following low MUFA beef consumption ($P > 0.05$). Aggregated post-beef HDL_3-S1P concentrations were significantly increased over aggregate pre-beef values ($P < 0.03$). The concentration of HDL_3-S1P in our study had an overall average concentration of 151 nmol/L, comparable to earlier reports (179).

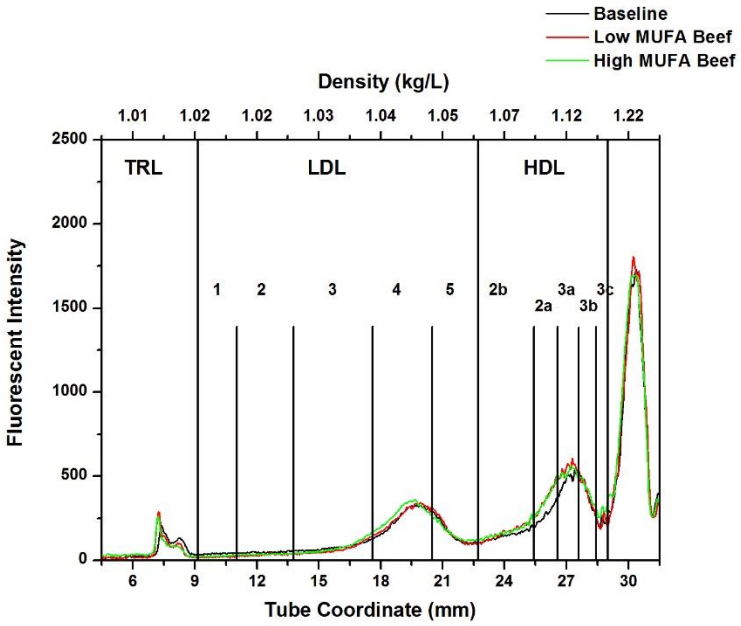


Figure 2.1 Lipoprotein density profile for one responder at baseline and after two beef diets.

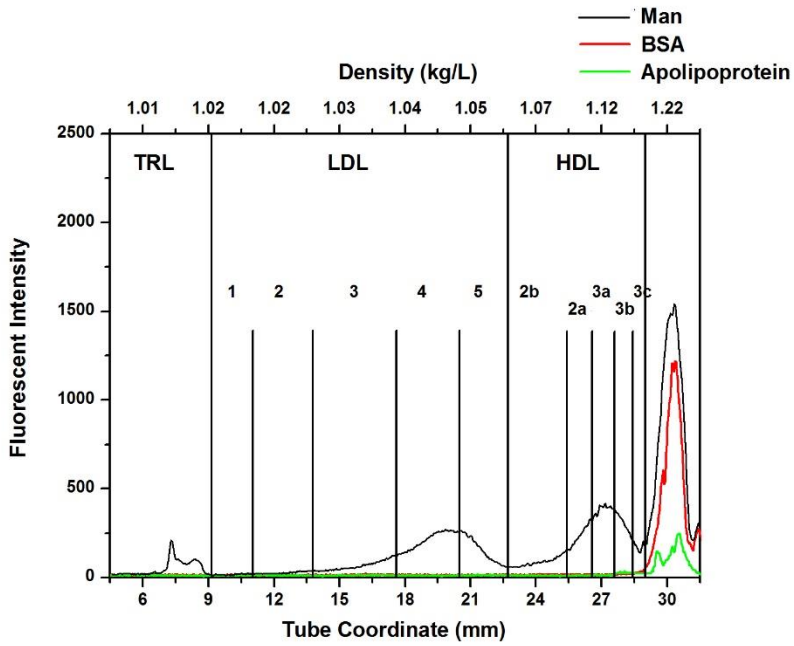


Figure 2.2 Density profiles generated using healthy man serum, BSA and apolipoprotein mixture.

Table 2.3 Effect of freeze thaw cycles on lipoprotein subfraction stability

Cycle	1	2	3
TRL	96 ± 5	88 ± 8	75 ± 6*
LDL_1	98 ± 15	113 ± 16	154 ± 34
LDL_2	91 ± 5	95 ± 5	103 ± 5
LDL_3	90 ± 3	95 ± 5	104 ± 5
LDL_4	92 ± 6	95 ± 5	103 ± 6
LDL_5	96 ± 4	96 ± 5	102 ± 6
HDL_{2b}	96 ± 2	96 ± 1	98 ± 4
HDL_{2a}	96 ± 2	97 ± 1	96 ± 2
HDL_{3a}	100 ± 3	99 ± 3	100 ± 5
HDL_{3b}	106 ± 6	109 ± 9	101 ± 6
HDL_{3c}	101 ± 9	101 ± 4	96 ± 4
Total LDL	99 ± 9	99 ± 3	102 ± 5
Total HDL	98 ± 2	98 ± 1	98 ± 3
HDL subfractions as % of HDL AUC			
HDL_{2b}	98 ± 2	99 ± 1	100 ± 2
HDL_{2a}	99 ± 1	99 ± 1	98 ± 1
HDL_{3a}	102 ± 2	101 ± 2	102 ± 3
HDL_{3b}	108 ± 5	111 ± 9	103 ± 8
HDL_{3c}	104 ± 10	103 ± 4	99 ± 4

Lipoprotein subfraction data after 1-3 freeze thaw cycles were calculated as percentages of their fresh compartments and expressed as mean ± SEM. n = 6. The effect of freeze thaw cycles were determined by paired t-tests. *P <0.05.

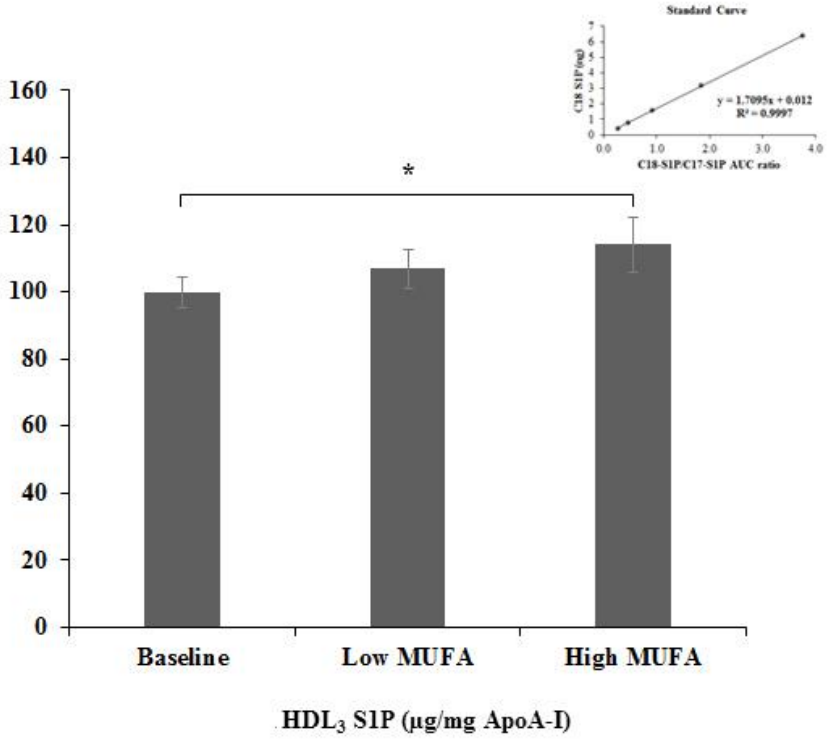


Figure 2.3 HDL_3-S1P before and after beef consumption. n = 25. C17-S1P was used as an internal standard during extraction procedure and S1P was calculated using the standard curve shown on the top right ($y = 1.7095x + 0.012$, $R^2 = 0.9997$ where y is the concentration of S1P and x is S1P AUC:C17-S1P AUC). HDL_3-S1P concentration was normalized to ApoA-I content. Effect of beef on S1P was determined by paired t-test.

Correlations of HOMA-IR and HDL_3-S1P with other ASCVD risk factors

Simple correlations were calculated for baseline and endpoint HOMA-IR and HDL_3-S1P and other analytes measured specifically for this study; additional correlations were calculated using total-C, TG, HDL-C, LDL-C and high sensitivity C-reactive protein (hsCRP) measured previously (162) (Table 2.4). HOMA-IR was most strongly and positively associated with TG and the fractional abundance of HDL_{3a} and HDL_{3c} (P <0.001); and conversely, negatively associated with the fractional abundance of HDL_{2b} (P <0.001). The correlations with the largest diameter subclass HDL_{2b} (r = -0.41, P <0.01) and smallest diameter subclass HDL_{3c} (r = 0.46, P <0.01) remained significant after adjustments for total HDL-C and TG. HOMA-IR was also positively correlated with hsCRP (P <0.01), resistin (P <0.01) and HDL_{3b} (P <0.05) and negatively correlated with HDL-C (P <0.01) and HDL_3-S1P (P <0.05). HDL_3-S1P was most strongly and positively correlated with total HDL-C and negatively with hsCRP, PAI-1 and resistin (P <0.001). To a lesser extent, S1P was negatively correlated with HDL_{3c} (P <0.01) and HDL_3 ApoA-I (P <0.05).

Correlations of beef induced changes in HOMA-IR and HDL_3-S1P with baseline ASCVD risk factors

Additional correlations to baseline ASCVD risk variables were calculated for the changes in HOMA-IR and HDL_3-S1P after beef intervention. Negative correlations were observed for the change in HOMA-IR with baseline HDL-C (P <0.05) and the change in HDL_3-S1P with baseline TG (P <0.001), total-C (P <0.01) and LDL-C (P <0.01) (Figure 2.4).

Table 2.4 Pearson correlation coefficients among HOMA-IR, HDL_3-S1P and serum ASCVD risk factors, HDL density distribution and HDL_3 apolipoprotein

Variable	HOMA-IR	HDL_3-S1P
Serum ASCVD risk factors		
HOMA-IR		-0.22*
TG	0.30***	-0.17
Total-C	0.12	0.22*
LDL-C	0.14	0.18
HDL-C	-0.25**	0.28***
hsCRP	0.24**	-0.29***
S-Hcy	0.09	-0.11
PAI-1	0.19	-0.35***
Adiponectin	-0.12	0.03
Resistin	0.24**	-0.37***
PON1	-0.15	0.05
HDL density distribution (% of total HDL AUC)		
HDL_{2b}	-0.44***	0.21*
HDL_{2a}	-0.19	0.14
HDL_{3a}	0.36***	-0.16
HDL_{3b}	0.20*	-0.17
HDL_{3c}	0.37**	-0.22*
HDL_3 composition		
ApoA-I	-0.07	-0.26**
ApoA-II	-0.14	-0.10
ApoE	0.01	0.03
ApoE/ApoA-I	0.02	0.13

Values are Pearson correlation coefficients. Outliers were tested by Cook's D Distance and 0-3 outliers were excluded for each correlation test. n = [97, 100] *P <0.05; **P <0.01; ***P <0.001.

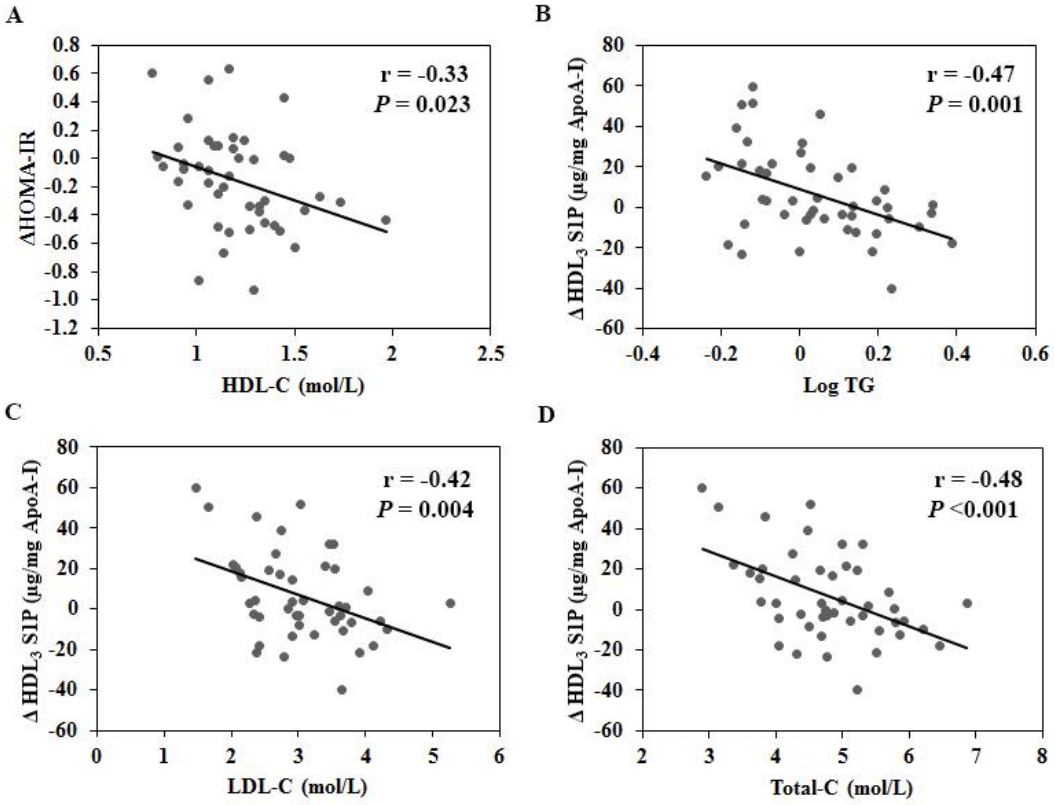


Figure 2.4 Correlations between 5 wk beef intervention induced changes in HOMA-IR with baseline HDL-C (A) and changes in HDL_3-S1P with baseline TG (B), LDL-C (C) and total-C (D).

Discussion

Consumption of either type of ground beef reduced HOMA by ~20% without affecting HDL density profile or amounts of ApoA-I, ApoA-II, Apo-E in HDL_3, or PON1 activity in plasma or markers of systemic inflammation and obesity. However, consumption of MUFA rich ground beef significantly increased HDL_3-S1P, suggesting that this form of beef enhanced HDL functionality. Functional HDL is defined by its ability to carry out RCT as well as other atheroprotective actions such as suppression of inflammation, thrombosis, apoptosis and oxidation in the vascular wall while promoting

vasodilation and resistance to infection (166). Effective RCT forms mature HDL_{2b} particles and this component of HDL is most often associated with reduced ASCVD risk (180).

The uniform improvement in insulin sensitivity by both beef diets in the present study may indicate that the beneficial effects were at least partially due to lean components of the meat, and perhaps improved dietary protein quality (165) as total dietary protein intake did not change (162). One possible explanation for the lack of a more beneficial outcome MUFA rich beef consumption was that by design, the MUFA:SFA was chosen to match the range of beef compositions commonly available in retail stores (162). Beef is a major contributor to MUFA intake in US (181, 182) and beef is most commonly consumed as ground beef (159). Other studies have created differences in daily MUFA intake of more than 13 g, much larger than the ~3g increase tested here. Moreover, some studies used combinations of dairy fat rather than intrinsic beef fat (183) to modify fat intake, and tested MUFA/SFA effects in diet with fat contents lower than 37% of energy rather than the 39% of energy used here (184) complicating direct comparisons.

HDL density profile was measured using NBD-ceramide labeled lipoprotein particles. Unlike chemical measurement of HDL-C, this method reflects overall particle mass density distribution expressed as AUC for density-defined regions. Serum samples used in the present study were thawed once. Freeze thaw cycles will alter some lipid measurements (185, 186), but 3 freeze-thaw cycles did not affect HDL density distribution or total AUC; albeit TRL AUC was significantly reduced. Total HDL AUC

was increased by low MUFA beef consumption by ~3.4%, while the 1.8% increase observed following high MUFA beef consumption was not significant. Interestingly, previous chemical measurement on replicate samples from this same study showed that HDL-C was only significantly elevated by MUFA rich beef consumption (162). Cholesterol comprises about 15% of HDL mass, and its chemical measurement in conjunction with density distribution information might assess HDL functionality in regards to RCT if an increase in HDL_{2b} AUC were observed. In this study, neither beef intervention caused changes to HDL density distribution. However, correlation analysis confirmed that HDL density subclasses were variably associated to ASCVD risk factors in a manner consistent with prior studies using beef as a protein source (160, 165, 183) as well as earlier prospective studies conducted on HDL subclass distribution and insulin sensitivity (169, 187, 188), indicating that the density distribution measurements are able to capture known physiological associations. Thus a question develops regarding what besides cholesterol contributed to the greater HDL AUC increase seen following low MUFA beef consumption.

Compositional change is a major cause for dysfunction within small and dense HDL. One study using shot-gun proteomics found ApoE enrichment in HDL_3 from cardiovascular patients (172). HDL_3 apolipoprotein composition was not influenced by 5 wk consumption of either beef type, nor did HDL_3 ApoE content correlate with HDL_3-S1P, HOMA-IR or other inflammatory markers. Enrichment of HDL with the bioactive lipid S1P is thought to confer numerous atheroprotective effects (12, 13, 189). HDL_3-S1P concentration increased after 5 wk of MUFA-rich beef consumption, suggesting an

improvement in HDL_3 functionality with consumption of this beef type. While only weak or non-significant correlations were observed for total HDL_3-S1P and plasma TG, LDL-C and total-C, the magnitude of increase in HDL_3-S1P following ground beef consumption was strongly correlated with baseline values of these variables. Low baseline values for plasma TG, LDL-C and total-C were associated with the greater increases in HDL_3-S1P post-beef consumption. The average tabular values may not adequately characterize the response of individuals.

Metabolic inflexibility (190) as indicated by an elevated HOMA-IR value, was associated with increased proportions of the smallest and most dense HDL_{3c} subfraction and decreases in the large HDL_{2b} particle associated with efficient RCT. Interestingly, ApoM is reported to be the sole carrier of S1P, and this apoprotein is found primarily in HDL_3 (62), however, ApoM is displaced towards TRL during postprandial lipemia and in dyslipidemic subjects (191, 192). In the current sample set HDL_3-S1P content was negatively associated with a fractional increase in the smallest and most dense HDL_{3c} subfraction, a subfraction whose increase was in turn associated with increases in HOMA-IR, suggesting that S1P did not associate with HDL_3 lipoprotein material formed in metabolically inflexible individuals; i.e. most likely dysfunctional non-ApoM containing HDL. Redistribution of ApoM to TRL may blunt S1P benefit mediated by the HDL activated SR-BI receptor as it is reported to co-operate with S1P receptors (12). Formation and lipolysis of triacylglycerol-rich HDL or failed RCT-mediated conversion of HDL_3 into HDL_2 due to dyslipidemia and inflammation could increase the abundance of HDL_3 lacking S1P. We found that HDL_3-S1P inversely correlated with the

prothrombotic or inflammatory markers of PAI-1 resistin and hsCRP, consistent with reported general anti-inflammatory properties of HDL_3-S1P (12, 13).

Limitations of the present study include a small sample size and free-living conditions of subjects that could compromise rigor of dietary adherence. Diet records (193) indicate subject intakes of folic acid, vitamin B6 and vitamin B12, that averaged 84%, 107% and 100% of the RDA for men 18-70 years old (194). These intakes are consistent with unchanged concentrations of the putative oxidative stressor homocysteine despite beef being a rich source of its parent molecule methionine. Less well-nourished individuals might differ in their response to beef intake. We did not conduct glucose tolerance tests and none of our subjects presented with overt metabolic syndrome and so additional studies looking specifically at this metabolic parameter would be desirable. Overall diet quality, particularly as it relates to risk for increases in metabolic inflexibility should be considered when evaluating the nutritional impacts of individual diet components. It would also have been desirable to genotype subjects, albeit the cross-over design insured that all subjects participated in each test diet. Never the less genetic information may have provided additional insight into observed responses (195).

Subject health status influenced HDL response to ground beef consumption as changes in HOMA-IR and HDL_3-S1P were associated with subject baseline plasma lipid profile (162). Beneficial changes to measures of HDL quality appear to occur in response to lean components of beef and to be amplified by an increased MUFA:SFA.

CHAPTER III

GROUND BEEF CONSUMPTION ALTERS HDL OXYLIPIN PROFILE IN HEALTHY MEN

Introduction

Oxylipins are a structurally and functionally diverse array of lipid mediators derived from PUFAs. Over the last decade, oxylipins have emerged as important regulators of inflammatory progress and have been implicated in various inflammatory conditions including type II diabetes, cardiac surgery, proteinuric hyperlipidemia and Immunoglobulin A nephropathy (76-79). Oxylipins can be generated either via auto-oxidation by ROS or via the actions of three enzyme families including COX, LOX, and CYP (80). Unlike COX derived oxylipins, that mainly occur in free forms, over 90% of LOX and CYP derived oxylipins in plasma are esterified in lipoproteins, with HDL containing the highest load for alcohols, epoxides and total oxylipins (90). Although it's still unclear whether HDL oxylipins are mainly targeted for hepatic disposal or they can be released and act as lipid mediators, recent studies show that the unique HDL oxylipin profile can serve as a potential biomarker for inflammation and vascular health. In chronic inflammatory mice models, HDL oxylipin profiles were shown to possess increased pro-inflammatory oxylipin moieties including 5-HETE and 9-HETE, which are biomarkers for 5-LOX activation and auto-oxidation respectively (78). CYP derived epoxides are potent vasodilators. One recent study reported that increased HDL LA and

ALA derived epoxides are associated with improvements in microvascular function (196).

Oxylipin profile is influenced by background diet, as dietary fats are modulators for tissue parent PUFA composition and the activities of the COX, LOX and CYP enzymatic pathways. One study investing the acute effects of high-fat diets on circulating oxylipins showed that a high MUFA diet increased postprandial CYP mediated oxylipins while high n-3 diet increased EPA and DHA derived oxylipins (197). In healthy human, supplementation of n-3 PUFA ethyl ester (P-OM3) resulted in elevated plasma n-3 PUFA derived oxylipins and reduced n-6 PUFA derived oxylipins (198). Interestingly, dietary fats appeared to have a differential effect on oxylipins in different lipoprotein compartments. Compared with AA derived oxylipins in LDL and VLDL, AA-oxylipins in HDL was less influenced by P-OM3 administration in hypertriglyceridemic, statin-treated subjects (199). On the other hand, HDL oxylipins have been reported to be more responsive to increased PUFA intake induced by walnut consumption than VLDL and LDL oxylipins in hypercholesterolemic women (196).

As MUFA has been suggested to be more beneficial than SFA and that MUFA:SFA has a wide range in beef, our previous study evaluated the effect of MUFA:SFA ratio of beef fat on plasma lipids in 27 healthy free-living men. We reported that the consumption of 5, 114 g ground-beef patties containing 24% total fat of either high or low MUFA:SFA per week for 4 wk both resulted in an elevation in total and relative plasma AA concentrations despite an increase in HDL-C (162). It remains unknown whether the increased plasma AA or beef consumption is associated with

elevated ASCVD risk by giving rise to pro-inflammatory lipids. In the present study, we evaluated the effect of 24% total fat beef consumption on HDL oxylipin profile.

Methods

Subjects and design

Banked plasma samples from 25 healthy men that successfully completed a randomized, cross-over beef feeding trial were used for the present study. The complete study design has been published (162). Briefly, the free-living participants consumed each of low MUFA and high MUFA beef diet for 5 wk in a random order with a 4wk wash out period between the diets. Each individual consumed five 114 g ground beef patties per week during the beef intervention period. The high MUFA ground beef patties, providing a MUFA:SFA = 1.10 were prepared from corn-fed Angus steers while the low MUFA beef patties, providing a MUFA:SFA = 0.71 were prepared from pasture-fed Angus steers. The total fat content of both types of patties was formulated to be 24% by weight using fat and lean trims. During the feeding period, subject total fat intake increased an average of 10 g/d, increasing dietary fat energy percentage from 38% to 39.5% and total energy intake by 6.5% on average. Despite the slight increase in energy intake, no change in bodyweight for subjects was observed at the endpoint. Blood samples were collected from fasted men at the start and end of each feeding period. Isolated plasma samples were stored at -80°C prior to analysis.

HDL oxylipin analysis

HDL (d = 1.063-1.21 g/mL) was isolated from plasma by NaCl-NaBr sequential flotation ultracentrifugation and then sealed in nitrogen and stored at -20°C until

extraction. HDL esterified fatty acids (EFA) and oxylipins were extracted by solid phase extraction (200) and quantified by liquid chromatography-tandem mass spectrometry (76) as previously described with modifications (83). Briefly, 0.2 mg Na_2EDTA/BHT was added to 250 µL of isolated HDL as anti-oxidants. The mixture was spiked with a set of deuterated oxylipin surrogates and subjected to hydrolysis in anhydrous sodium methoxide for 2 h at 60 ºC. The hydrolyzed lipids were diluted with 1 mL 95:5 (v/v) water:methanol with 0.1% acetic acid and then loaded onto the 60mg Oasis HLB SPE cartridges (Waters Corp, Milford MA) by gravity and air dried. The columns were wetted with 0.5 mL methanol and eluted with 2 mL ethyl acetate into polypropylene tubes containing 6 µL 30% glycerol in methanol. Samples were evaporated by centrifugal vacuum, reconstituted in 50 µL of methanol containing 100 nM of the quality control 1-cyclohexyl-ureido-3-dodecanoic acid (CUDA) and filtered with Durapore® PVDF 0.1 µm spin-filter tubes (Millipore, Billerica, MA) at 4°C. Then the extracted lipids were separated by reverse phase ultra-performance liquid chromatography (UPLC) using a 2.1 × 150 mm, 1.7 µm Acquity EBH column (Waters Corp, Milford MA). Analytes were detected on an ABI 4000QTrap (Applied Biosystems Inc., Foster City, CA) by multiple reaction monitoring (MRM) after negative mode electrospray ionization, tandem quadrupole mass spectroscopy (Micromass, Manchester, UK).

Statistics

Data analysis was performed using JMP 11.0 (SAS Institute Inc, Cary, NC). The distributions of variables were assessed by Shapiro-Wilk test and log transformations of skewed variables were used in subsequent analyses. HDL EFA and oxylipins at baseline

and after the consumption of low or high MUFA beef were compared by paired Student's t test. Simple correlations were tested by Pearson or Spearman's correlation coefficient. Correlation network was visualized by Cytoscape (201). The level of significance was $P < 0.05$.

Results

The present study was a secondary analysis of the original hamburger patty feeding study, with the complete feeding design published previously (162). A total of 48 oxylipin compounds were detected in HDL. Their PUFA precursors included the n-6 PUFA LA, DGLA and AA and the n-3 PUFA ALA, EPA and DHA. None of the concentrations of these EFA in HDL was altered after 5 wk consumption of both ground beef patties (Table 3.1).

Overall HDL oxylipin distribution

HDL oxylipins were primarily n-6 PUFA derivatives with ~75% oxylipins derived from LA, ~21% derived from AA and ~4% from n3 fatty acids, reflecting the distribution pattern of their parent PUFA (Table 3.2). LOX and CYP pathway represent two major lipid oxygenation pathways. Among all oxylipins, alcohols and their ketone metabolites from the LOX pathway dominated in concentration, followed by epoxides from the CYP pathway. The sEH metabolites diols and triols comprised less than 2% of total HDL oxylipins. While the concentration of total oxylipins remained unchanged after both beef interventions, a ~18% increase in the fractional abundance of DHA derived oxylipins was induced by both low ($P = 0.02$) and high MUFA ($P < 0.001$) beef

patties. High MUFA beef also caused a ~2% decrease in total LA derived oxylipins (P = 0.02) and a ~7% increase in AA derived oxylipins (P = 0.02).

Table 3.1 HDL EFA composition at baseline and after 5 wk beef intervention

FA type	FA (μMol/100μMol EFA)	Baseline	Low MUFA	High MUFA
SFA	16:0	23.7 ± 0.4	23.5 ± 0.4	23.6 ± 0.3
	18:0	9.5 ± 0.1	9.4 ± 0.1	9.4 ± 0.1
MUFA	16:1 n7	1.1 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
	18:1 n9	12.7 ± 0.3	12.4 ± 0.3	12.7 ± 0.3
PUFA	18:2 n6	36.3 ± 0.7	37.1 ± 0.9	36.5 ± 0.8
	18:3 n6	0.41 ± 0.03	0.37 ± 0.03	0.34 ± 0.02
	18:3 n3	0.35 ± 0.02	0.36 ± 0.03	0.34 ± 0.02
	20:3 n6	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
	20:4 n6	11.1 ± 0.3	11.0 ± 0.3	11.6 ± 0.4
	20:5 n3	0.70 ± 0.09	0.71 ± 0.12	0.57 ± 0.05
	22:6 n3	2.0 ± 0.2	2.0 ± 0.2	2.1 ± 0.2

Data were expressed as mean ± SEM. n = 25. HDL EFA composition before and after beef intervention were compared by paired t-test. n3 PUFA were log transformed before statistical analysis.

Table 3.2 Overall HDL oxylipin profile at baseline and after 5 wk beef intervention

	Baseline	Low MUFA	High MUFA	Low MUFA		High MUFA	
				Fold change	*P*	Fold change	*P*
Total oxylipids (nM)	11468 ± 795	10613 ± 577	10497 ± 646		0.28		0.16
% C18:2	76 ± 1	75 ± 1	74 ± 1		0.23	-2.1%	0.02
% C18:3	0.80 ± 0.04	0.80 ± 0.04	0.78 ± 0.04		0.96		0.53
% C20:3	0.39 ± 0.02	0.39 ± 0.04	0.38 ± 0.03		0.47		0.47
% C20:4	21 ± 1	21 ± 1	22 ± 1		0.44	7.1%	0.02
% C20:5	0.49 ± 0.06	0.58 ± 0.10	0.46 ± 0.06		0.71		0.43
% C22:6	1.6 ± 0.2	1.8 ± 0.3	1.8 ± 0.2	17.4%	0.02	18.6%	<0.01
% Epoxide	23 ± 1	25 ± 1	25 ± 1		0.14		0.24
% Diol	0.82 ± 0.05	0.96 ± 0.14	0.73 ± 0.05		0.33	-7.7%	0.05
% Triol	0.35 ± 0.04	0.36 ± 0.10	0.24 ± 0.03		0.35	-9.6%	0.03
% Hydroperoxide	0.16 ± 0.01	0.17 ± 0.01	0.16 ± 0.01		0.33		0.57
% Alcohol	43 ± 2	41 ± 2	42 ± 2		0.69		0.23
% Ketone	33 ± 1	33 ± 1	32 ± 1		0.25		0.71
Diol:Epoxide	0.037 ± 0.003	0.041 ± 0.008	0.031 ± 0.003		0.83		0.02
Ketone:Alcohol	0.81 ± 0.05	0.88 ± 0.08	0.82 ± 0.07		0.63		0.77

Distinct oxylipin classes were expressed as mean ± SEM. n = 25. Baseline and endpoint values were compared by paired t-tests. Fold changes were calculated as 100%*(endpoint-baseline)/baseline.

Individual HDL oxylipin distribution

The concentrations for individual HDL oxylipins at baseline and feeding endpoint were presented in Table 3.3. High MUFA beef consumption significantly reduced a number of LA derived oxylipins including the hydroperoxides 9-HpODE and 13-HpODE, the diols 9,10-DiHOME and 12,13-DiHOME, the triol 9,12,13-TriHOME and the ketones 9-KODE, 13-KODE and EKODE. The metabolic pathway for these LA-oxylipins were shown in Figure 3.1. High MUFA beef also decreased AA-ketone 5-KETE and EPA-epoxide 14(15)-EpETE while the concentration of DHA-epoxide 19(20)-EpDPE increased. The fold changes from baseline for these oxylipins induced by high MUFA beef ranged from 10-23%. In contrast, low MUFA beef only decreased the concentration of 5-KETE by ~12% with no effect observed on other individual oxylipins. To examine whether the changes in the concentration of oxylipins were caused by possible alteration in the activities of sEH and hydroxyl fatty acid dehydrogenase, ratios of ketones:alcohols and diols:epoxides were calculated. Results showed that high MUFA beef significantly decreased the ratio of diol:epoxide from 0.037 to 0.031 when compared with baseline. While high MUFA beef consumption appeared to have a more profound effect on HDL oxylipin profile, no significant difference was observed for all measured oxylipin variables after two beef diets.

Table 3.3 Individual HDL oxylipins at baseline and after 5 wk beef intervention

PUFA	Type	Oxylipin (nM)	Baseline	Low MUFA	High MUFA	Low MUFA		High MUFA	
						Fold change	*P*	Fold change	*P*
18:2	Epoxide	9(10)-EpOME	814 ± 44	827 ± 48	824 ± 61		0.78		0.80
	Epoxide	12(13)-EpOME	903 ± 56	949 ± 61	948 ± 78		0.35		0.84
	Diol	9,10-DiHOME	69 ± 6	76 ± 13	55 ± 3		0.89	-12.9%	0.02
	Diol	12,13-DiHOME	7.6 ± 0.6	7.9 ± 1.2	5.4 ± 0.4		0.67	-23.3%	<0.01
	Hydroperoxide	9-HpODE	4.0 ± 0.2	3.8 ± 0.3	3.4 ± 0.2		0.86	-12.3%	0.01
	Hydroperoxide	13-HpODE	1.6 ± 0.1	1.5 ± 0.1	1.4 ± 0.1		0.72	-10.9%	0.02
	Alcohol	9-HODE	835 ± 87	742 ± 73	747 ± 73		0.25		0.39
	Alcohol	13-HODE	2961 ± 327	2471 ± 209	2479 ± 225		0.12		0.20
	Ketone	EKODE	131 ± 8	117 ± 5	112 ± 8		0.21	-12.6%	0.01
	Ketone	9-KODE	374 ± 28	323 ± 15	307 ± 18		0.10	-13.9%	0.01
	Ketone	13-KODE	2582 ± 167	2388 ± 153	2249 ± 134		0.22	-10.3%	0.01
	Triol	9,12,13-TriHOME	23 ± 4	20 ± 5	12 ± 1		0.23	-10.7%	0.02
	Triol	9,10-13-TriHOME	19 ± 3	16 ± 4	12 ± 1		0.29		0.08
18:3	Epoxide	9(10)-EpODE	33 ± 2	32 ± 3	31 ± 2		0.39		0.08
	Epoxide	12(13)-EpODE	4.4 ± 0.4	4.3 ± 0.5	4.2 ± 0.4		0.90		0.38
	Epoxide	15(16)-EpODE	31 ± 2	32 ± 3	30 ± 3		0.96		0.42
	Alcohol	9-HOTE	11 ± 1	9 ± 1	8 ± 1		0.10		0.07
	Alcohol	13-HOTE	11 ± 2	9 ± 1	9 ± 1		0.06		0.16
20:3	Alcohol	15-HETrE	44 ± 4	43 ± 6	40 ± 5		0.29		0.23
20:4	Epoxide	8(9)-EpETrE	100 ± 7	97 ± 6	100 ± 7		0.77		0.81
	Epoxide	11(12)-EpETrE	252 ± 14	244 ± 14	250 ± 16		0.58		0.74

Table 3.3 Continued

PUFA	Type	Oxylipin (nM)	Baseline	Low MUFA	High MUFA	Low MUFA		High MUFA	
						Fold change	*P*	Fold change	*P*
20:4	Epoxide	14(15)-EpETrE	257 ± 17	265 ± 18	281 ± 20		0.63		0.21
	Diol	5,6-DiHETrE	7.7 ± 0.5	7.1 ± 0.4	7.1 ± 0.5		0.18		0.26
	Diol	8,9-DiHETrE	3.4 ± 0.2	3.4 ± 0.2	3.5 ± 0.2		0.74		0.71
	Diol	11,12-DiHETrE	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.1		0.98		0.92
	Diol	14,15-DiHETrE	1.0 ± 0.1	1.0 ± 0.1	0.9 ± 0.1		0.45		0.15
	Hydroperoxide	5-HpETE	0.09 ± 0.01	0.08 ± 0.01	0.08 ± 0.00		0.98		0.09
	Hydroperoxide	12-HpETE	1.1 ± 0.1	1.1 ± 0.1	1.0 ± 0.0		0.45	-9.2%	0.04
	Hydroperoxide	15-HpETE	3.2 ± 0.2	3.1 ± 0.2	2.9 ± 0.2		0.71		0.11
	Prostaglandin	PGB2	5.2 ± 0.8	4.4 ± 0.8	4.6 ± 0.8		0.20		0.33
	Leukotriene	LTB4	3.4 ± 0.3	4.1 ± 0.7	3.8 ± 0.4		0.72		0.93
	Alcohol	5-HETE	300 ± 41	240 ± 26	265 ± 30		0.10		0.43
	Alcohol	8-HETE	116 ± 11	120 ± 15	131 ± 20		0.81		0.72
	Alcohol	9-HETE	102 ± 11	102 ± 13	113 ± 17		0.63		0.67
	Alcohol	11-HETE	165 ± 17	173 ± 22	185 ± 28		0.92		0.77
	Alcohol	12-HETE	142 ± 14	144 ± 18	158 ± 24		0.55		0.87
	Alcohol	15-HETE	248 ± 20	244 ± 27	256 ± 29		0.35		0.78
	Ketone	5-KETE	83 ± 6	67 ± 5	69 ± 5	-12.2%	0.02	-12.4%	0.01
	Ketone	12-KETE	268 ± 17	256 ± 17	249 ± 16		0.46		0.19
	Ketone	15-KETE	267 ± 17	253 ± 16	246 ± 16		0.43		0.17
20:5	Epoxide	14(15)-EpETE	15 ± 2	16 ± 4	12 ± 2		0.79	-9.9%	0.04
	Epoxide	17(18)-EpETE	28 ± 5	31 ± 9	24 ± 4		0.95		0.21

Table 3.3 Continued

PUFA	Type	Oxylipin (nM)	Baseline	Low MUFA	High MUFA	Low MUFA		High MUFA	
						Fold change	*P*	Fold change	*P*
20:5	Alcohol	5-HEPE	24 ± 4	24 ± 5	19 ± 4		0.59		0.09
	Alcohol	12-HEPE	15 ± 3	16 ± 4	13 ± 3		0.88		0.19
	Alcohol	15-HEPE	20 ± 4	22 ± 3	20 ± 4		0.50		0.64
22:6	Epoxide	16(17)-EpDPE	44 ± 6	50 ± 7	47 ± 5		0.09		0.28
	Epoxide	19(20)-EpDPE	56 ± 8	63 ± 11	65 ± 10		0.15	14.2%	0.03
	Alcohol	17(R)-HDoHE	74 ± 10	80 ± 16	87 ± 17		0.55		0.99

HDL oxylipin concentrations were expressed as mean ± SEM. n = 25. Values at baseline and after beef consumption were compared by paired t-tests. Fold change was calculated as 100%*(endpoint-baseline)/baseline.

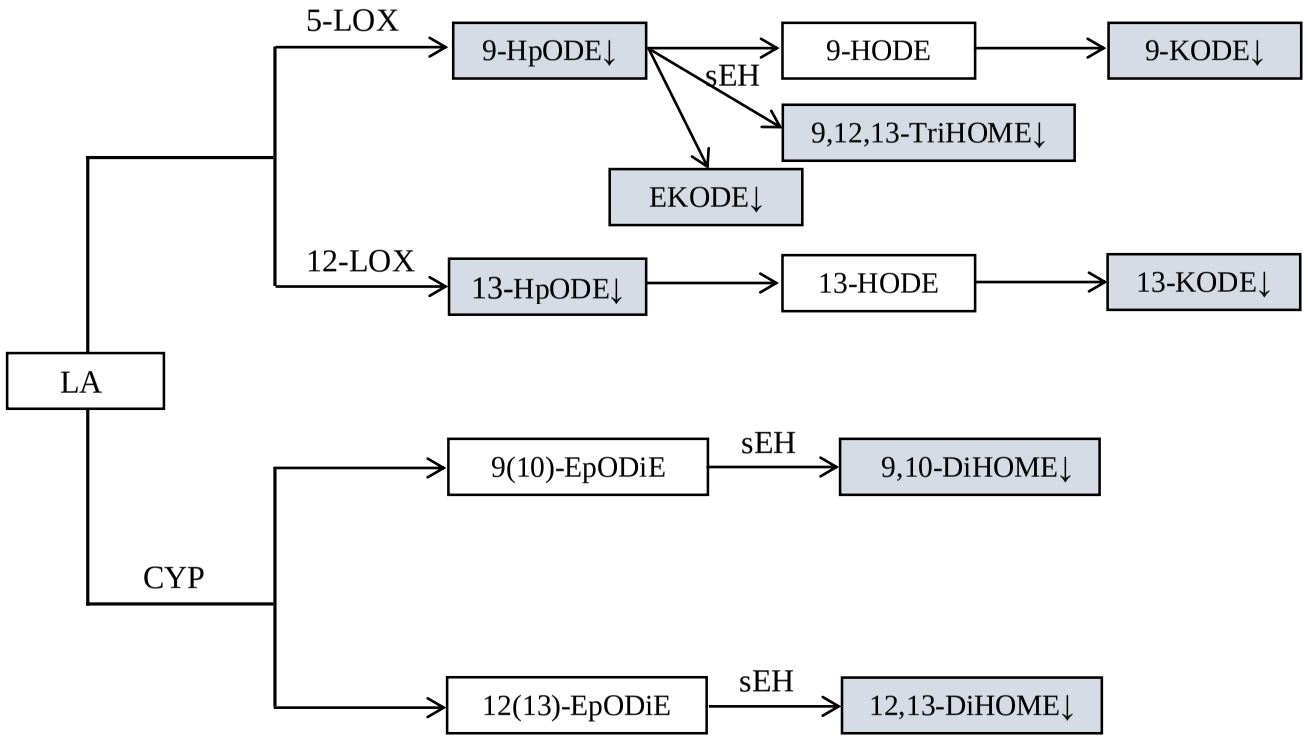


Figure 3.1 Metabolic pathway for LA derived oxylipins and their concentration changes in HDL after 5 wk high MUFA beef intervention. Gray shaded metabolites were significantly decreased.

Correlations between changes in HDL oxylipins and ASCVD risk factors

In our previous study, we reported that beef consumption non-differentially raised HDL_3-S1P and improved insulin sensitivity in the same study population. Correlation analysis showed that a greater increase in HDL_3-S1P was associated with a greater reduction in HDL 5-KETE after beef consumption (Figure 3.2). No correlation was observed for the change in insulin sensitivity with any change in HDL oxylipins (data not shown).

Correlations between changes in HDL oxylipins and baseline HDL EFA

As background diet, especially n3 fat intake, may affect oxylipin formation, we analyzed the correlation between baseline HDL EFA and beef induced changes in oxylipins. No effect of baseline HDL SFA, MUFA or n-6 PUFA on concentration change in oxylipins was observed. However, the magnitude of changes for half of the oxylipins whose concentration was influenced by beef consumption was associated with background HDL n-3 PUFA. All significant correlations are shown in Figure 3.3. Baseline ALA was negatively associated with LA derived diols 9,10-DiHOME (P = 0.040) and 12,13-DiHOME (P = 0.002). Baseline EPA was associated positively with the change in the AA ketone metabolite 5-KETE (P = 0.008) and negatively with the change in AA epoxide metabolite 14(15)-EpETE (P = 0.003) while baseline DHA was positively correlated with the changes in LA ketone metabolites EKODE (P = 0.036) and 13-KODE (P = 0.037).

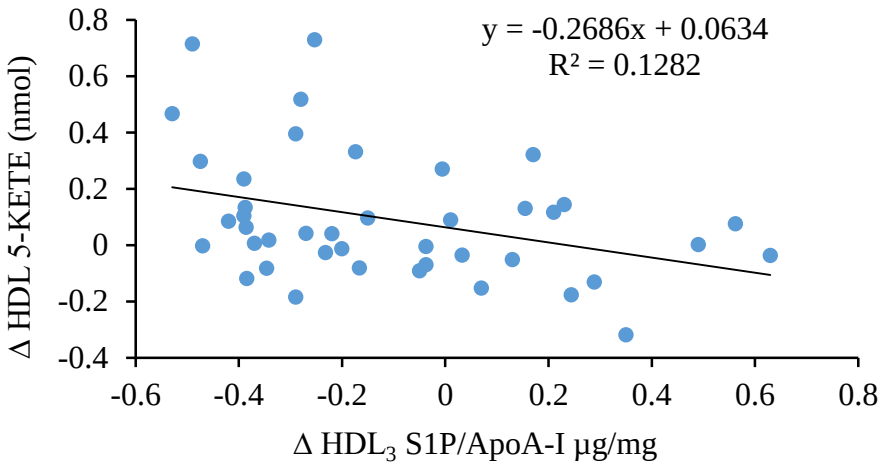


Figure 3.2 Pearson correlation between the changes in HDL_3-S1P and HDL 5-KETE by beef consumption.

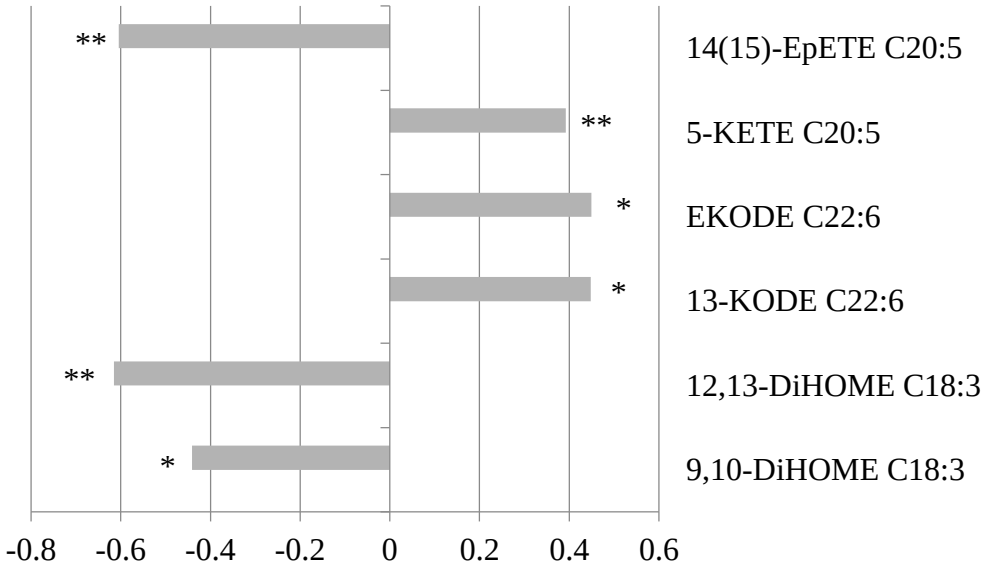


Figure 3.3 Spearman correlation between baseline HDL EFA and beef induced changes in HDL oxylipins. Changes (endpoint-baseline) in 5-KETE included data from both low and high MUFA beef interventions. Changes in other oxylipins only included data from high MUFA intervention. *P <0.05; **P <0.01.

As no significant differences were observed for HDL oxylipins after the two beef diets, data were combined for correlation analysis. Pearson correlation matrix was calculated for oxylipin metabolites (Figure 3.4). As shown by the heat map, oxylipin regioisomers generally shared similar correlations with other oxylipins. For summarization and visualization purpose, oxylipins were grouped according to oxylipin class and their parent fatty acids, with median correlation coefficients within the groups of related metabolites calculated (Figure 3.5). At baseline, LOX pathway products were highly interdependent. Hydroperoxides were strongly correlated with all the down-streaming LOX products derived from n6 PUFA including alcohols (r = 0.41, (0.34, 0.49), median (1^{st} quantile, 3^{rd} quantile)), ketones (r = 0.74, (0.66, 0.87)), and triols (r = 0.39, (0.38, 0.42)). n6 PUFA derived alcohols were strongly associated with the down-streaming ketones (r = 0.50, (0.44, 0.60)) and triols (r = 0.48, (0.39,0.68)), as well as n3 PUFA derived alcohols (r = 0.62, (0.52, 0.70)). Relatively weaker correlations were observed between LOX pathway products and CYP pathway products. The median correlation coefficients for n6 PUFA derived epoxides with n6 PUFA derived hydroperoxides, alcohols, ketones were r = 0.39 (0.28, 0.47), r = 0.25 (0.18, 0.34), r = 0.30 (0.25, 0.34) respectively. The beef diet intervention greatly disrupted the interdependencies between LOX pathway products. The only strong correlations remained after beef consumption were the correlations between n6 PUFA derived hydroperoxides and ketones (r = 0.68, (0.60, 0.79)), and the correlations between n6 and n3 PUFA derived alcohols (r = 0.73, (0.68, 0.83)). On the other hand, the correlations between LOX and CYP products were relatively less influenced. The correlations

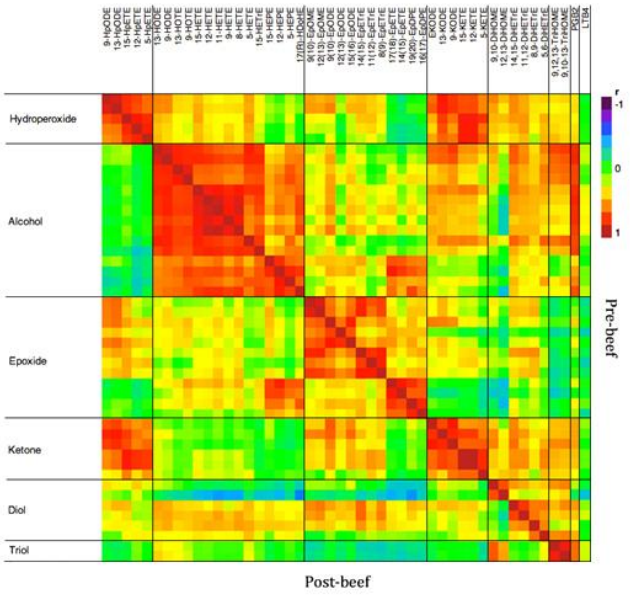


Figure 3.4 Pearson correlation matrix for HDL oxylipin metabolites before (above the diagonal) and after (below the diagonal) 5 wk beef patty intervention. Correlations with a $|r| \geq 0.23$ were significant.

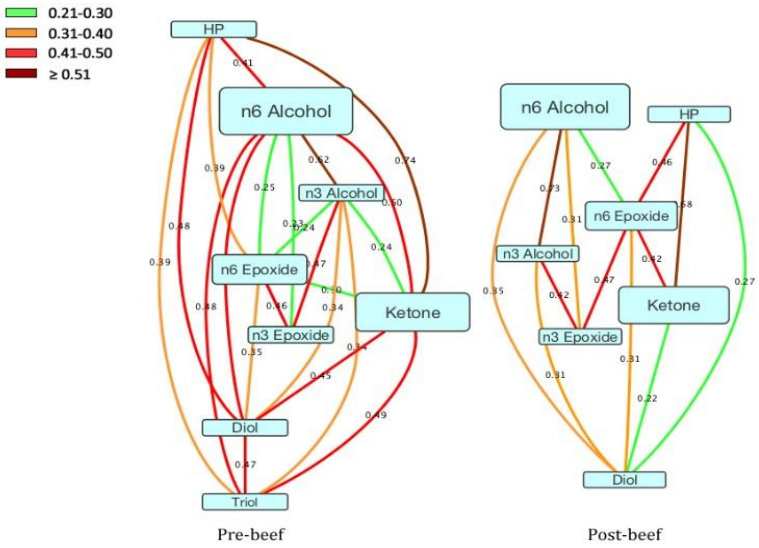


Figure 3.5 Correlation network for HDL oxylipin metabolites before and after 5 wk beef patty intervention. Median correlation coefficients within the groups of related metabolites were displayed. Only correlations with a median $|r| \geq 0.21$ were included in the network graph. Size of the rectangle reflected the relative abundance of the oxylipin group.

between n6 PUFA derived epoxides with n6 PUFA derived hydroperoxides and ketones appeared to slightly increased, from 0.39 (0.28, 0.47) to 0.46 (0.34, 0.52), and 0.30 (0.25, 0.34) to 0.42 (0.36, 0.47) respectively.

Discussion

Oxylipins are lipid mediators which play important roles in inflammation and immune homeostasis. HDL is a major carrier for circulating oxylipins and has displayed distinctive oxylipin profile compared with VLDL and LDL, suggesting it may provide a different predictive value for disease than total circulating oxylipins. Here we demonstrate for the first time that ground beef consumption decreases the pro-inflammatory 5-KETE in HDL particles in healthy individuals regardless of beef fatty acid composition and that oxylipin response to ground beef is influenced by baseline n3 status.

The AA derived 5-KETE is oxidized from 5-HETE by 5-hydroxyeicosanoid dehydrogenase (5-HEDH). 5-HEDH is expressed in various inflammatory and structural cells including neutrophils, monocytes and endothelial cells. 5-HEDH has extremely high substrate selectivity and is regulated by oxidative stress (202). The pro-inflammatory and oxidative stress induced chemoattractant 5-KETE is nearly 100 times more potent than 5-HETE and can stimulate eosinophil migration (203, 204). A reduction in HDL 5-KETE was induced by both high and low MUFA beef, suggesting this effect may be attributed to the lean portion of beef. Our previous study showed that 5 wk beef consumption significantly raised S1P in HDL_3 per se. S1P is a signaling molecular that mediated various HDL atheroprotective functions including inhibiting

ROS production and MCP-1 secretion from endothelium (12) as well as promoting NO-dependent vasorelaxation (16). Here we demonstrated that the reduction in HDL 5-KETE is inversely correlated with the increase in HDL_3 S1P. One possible underlying mechanism is that the activity of 5-HEDH requires NADP+ as a cofactor (203) and the inhibition of NADPH oxidase by HDL-S1P (12) may inhibit the enzymatic activity of 5-HEDH and thus decreasing the synthesis of 5-KETE. Interestingly, the auto-oxidation marker 9-HETE was not altered after both beef diets, indicating a differentiation between enzyme-mediated and auto-oxidation products. Our previous study showed that HOMA-IR was also altered by both beef diets. No correlation was observed between insulin sensitivity and oxylipin profile change in healthy men. This result in some way supported one study that investigated the associations between insulin sensitivity and postprandial oxylipin profile change and observed no significant association in healthy subjects (197). As the oxylipin profile in healthy controls varied considerably from diabetic patient, it's possible that there's a threshold effect of insulin sensitivity on oxylipin profile (93).

After high MUFA beef consumption, all detected HDL hydroperoxides showed a tendency to decrease with significant *P* values observed for LA-derived hydroperoxides and marginal *P* values observed for AA-derived hydroperoxides. HDL has been reported as the predominant carrier for plasma hydroperoxides in mice models of atherosclerosis (205) and human (206). While HDL contains various anti-oxidant enzymes with PON1 being able to eliminate peroxidized phospholipid, our previous data showed that PON1 activity was not altered after beef consumption. The decrease in hydroperoxides may

result from a decreased formation, which can be achieved either through down-regulation of LOX pathway or the reduction in the availability of reactive oxygen species (144). The fractional abundance of both diols and triols were reduced by high MUFA beef consumption. While diol oxylipins were known to be generated from the CYP pathway and triol metabolites from the LOX pathway, they are both formed through the direct action of sEH from respective epoxide and hydroperoxide precursors (207). The ratio of plasma DiHOMEs to EpOMEs has been reported as a reliable biomarker for sEH activity (208). In the present study, the ratio of HDL DiHOMEs to EpOMEs significantly decreased. However, it was unclear whether it was due to reduced cellular production of diols and triols or reduced retention of these oxylipins in HDL. It should be noted that the high MUFA beef induced reduction in the fractional abundance of diol was caused by the reduction in absolute HDL DiHOME concentration. LA-derived diols have been shown to possess cytotoxic effects and can cause vascular endothelial cell dysfunction (209, 210). A reduction in diol synthesis may imply atheroprotective effects. High MUFA beef consumption also diminished the concentrations of the less well studied LA-derived ketone metabolites 9-KODE, 13-KODE and EKODE in HDL. Plasma oxylipin profile in in type 2 diabetic patients displayed elevated EKODE and 13-KODE content (93).

Basal n3 FA status has been previously shown to modulate oxylipin response to n3 FA administration in both norm- and hyper-lipidemic healthy individuals (211, 212). Here we report that n3 FA status also influences HDL oxylipin response to beef diets, though the effect was not universal (ie. suppression of all n6 PUFA derived oxylipins).

Importantly, we observed a positive correlation between basal EPA and the reduction in 5-KETE. High dose DHA+EPA supplements have been reported to down-regulate the gene expression of ALOX5 in peripheral blood mononuclear cells (213). If overall 5-LOX pathway activity was already low in subjects with high basal n3 FA, a diminishing 5-KETE response may be expected as the subject was in optimal health. In fact, greater reduction was observed in subjects with higher baseline 5-KETE (data not shown). On the other hand, our results clearly showed that n3 FA of differing chain length had differing effects on oxylipin profile and thus provide evidence that composition of n3 PUFA may be of relevance to maximize the health benefits of n3 supplements.

The majority of previous diet-oxylipin relation studies are focused on the effect of parent PUFA manipulation on plasma oxylipin profile, especially by using n3 FA supplementation (83, 199, 211, 212, 214, 215). Our study was initially conducted as plasma total AA was increased after both beef diets. Interestingly, we observed no change in total HDL esterified AA or AA derived oxylipins, nor any significant correlation between the increase in plasma AA with the change in individual oxylipin class (data not shown). These observations suggest the effect of beef consumption on HDL oxylipin profile are independent of parent PUFA modulation. Human AA are distributed between tissue and membrane compartments with varying turnover rate and physiological function (216). Indeed, a series of human dietary AA supplementation study have showed that AA phospholipids in adipose tissue could remain unchanged despite a two-fold increase in plasma (217). Currently, little is known about the main tissue PUFA pool for oxylipin generation and the source for HDL oxylipins. AA

enrichment in meat product has been a health concern as they could give rise to pro-inflammatory lipid mediators. As HDL is the major AA oxylipin carrier in plasma (199), our study offer evidence that short-term beef consumption induced moderate AA intake increase may not translate into increased pro-inflammatory oxylipin production in healthy subjects. On the other hand, our study showed that beef consumption greatly reduced the interdependencies between LOX pathway products, possibly by inducing differing effect on down-steaming oxylipin metabolizing enzymes. Similar reduction of interdependency of HDL oxylipins was observed by P-OM3 administration in a previous study, illustrating of the complexity of oxylipin profile regulation (199).

In summary, this study showed that regular consumption of a serving of a moderate fat ground beef significantly decreased the pro-inflammatory 5-KETE in HDL in healthy subjects. This effect was independent of ground beef fatty acid composition, but was dependent of individual basal n3 FA status. MUFA content in high MUFA beef appear to have additional health benefits, but the effect is moderate as no significant difference was observed for endpoint oxylipin profile for low and high MUFA beef diets. The present study is a pilot study on dietary modulation of HDL oxylipins. Further knowledge about the kinetics of oxylipin formation, transportation and turnover (possibly with isotopic tracer studies) as well as genetic typing of the subjects will vastly improve our understanding of our results.

CHAPTER IV

METABOLIC INFLEXIBILITY LIMITS REDUCTIONS IN SMALL DENSE LDL DUE TO LEAN BEEF CONSUMPTION

Introduction

LDL particles are highly heterogeneous in terms of atherogenicity due to the variations in size, composition and physiochemical properties. Compared with larger LDL particles, small dense LDL (sdLDL) particles have a higher propensity to penetrate the artery wall and bind to proteoglycans (218), increased susceptibility to oxidation (219) and reduced affinity for the LDL receptor (220). Thus, sdLDL particles are an independent risk factor for CHD (221).

Intervention studies have shown that LDL density distribution is modulated by dietary macronutrient composition. Compared with an iso-caloric high fat diet, low fat/high carbohydrate diets are associated with either no change or a decrease in LDL particle size (222-226). The difference among studies could be due, in part, to differences in type and amount of carbohydrate used in the studies. Replacement of carbohydrate by protein (from plants or mixed sources) in moderate or high fat eucaloric diets is generally associated with an improved LDL density profile (225, 227). While soy, as a protein source, has been more extensively studied and shown to improve the LDL density profile (228, 229), little is currently known about the effect of animal protein (and different animal proteins) on LP density distribution. A high degree of within-study variation in individual LDL particle responsiveness has been consistently

reported, even under controlled feeding conditions, suggesting the influence of genetic and other environmental factors on the responsiveness of LDL particles. ApoE phenotype (222) and small LDL subclass pattern (226) have both been identified as determinants of LDL response to low fat diets.

The DASH dietary pattern is considered a gold standard heart healthy diet that features fruits and vegetables as well as reduced SFA, cholesterol and red meat (163, 164). While some epidemiological studies reported adverse health associations for red meat, this may be attributed to the SFA intake, varying levels of meat processing and additives, and/or residual confounding factors (145). To test whether lean beef could be included in a heart healthy diet, the BOLD study compared the cholesterol lowering effects of a DASH dietary pattern with two DASH-like dietary patterns that provided different amounts of lean beef when consumed by a mildly hypercholesterolemic population (165, 230). The primary outcome of that trial was the significant (and similar) reduction in total-C and LDL-C in the DASH and lean beef containing diets compared to the HAD control diet (165). In addition, the higher protein/higher lean beef diet was the only dietary intervention to elicit a significant reduction in systolic blood pressure compared to the control diet (230). The aim of this secondary analysis was to determine the effect of dietary patterns that varied in the amount of dietary protein provided by lean beef on LP density distribution and the prevalence of sdLDL. In addition, inflammatory status and insulin sensitivity have both shown to affect lipoprotein particle size modulation (231). The secondary aim was to assess whether baseline health status affected the dietary responsiveness of LP density distribution.

Methods

Subjects and design

The present study is a secondary analysis of 27 complete sample sets available from the original BOLD study. The detailed design has been published (165). Briefly, plasma samples were taken from healthy men and women (36-65 y of age) with moderately high baseline LDL-C (2.47-4.83 mmol/L). No lipid-lowering medications or dietary supplements were taken at baseline and during the study. The BOLD study evaluated the effects of four diets: HAD (control), DASH, BOLD and BOLD+ (Table 4.1). Subjects consumed each of the four test diets for 5 wk in a random order in a controlled-feeding setting with a minimum on a 1-wk compliance break between the diets. The HAD was higher in SFA and total fat. It also included more refined grains, added sugars, and full-fat dairy products compared to the test diets. The DASH, BOLD and BOLD+ diets included more whole grains, vegetables, fruits, and low-fat dairy products. The DASH and BOLD (28 g/d vs. 113 g/d lean beef) were matched for macronutrient energy distribution, but differed in protein source. BOLD+ was similar to BOLD and DASH, but contained greater amounts lean beef (153 g/d) and protein (27% vs. 18-19% energy, respectively). The total energy content of the test diets was individually adjusted to keep participants' weight stable during the feeding periods.

Table 4.1 Energy and macronutrient profiles of the study diets

	HAD	DASH	BOLD	BOLD+
95% lean Beef (g/day)	20	28	113	153
Calories (kcal)	2097	2106	2100	2104
Protein (%E)	17	18	19	27
Carbohydrate (%E)	50	55	54	45
Fat (%E)	33	27	28	28
SFA (%E)	12	6	6	6
MUFA (%E)	11	9	11	12
PUFA (%E)	7	8	7	7

Laboratory measurement

Insulin sensitivity was estimated by HOMA score (174) and calculated from previously measured plasma and insulin concentrations. LP density profile was determined by imaging of fluorescently stained lipoproteins following NaBiEDTA ultracentrifugation as previously described with modifications (232). Briefly, 6 µL plasma was incubated with 10 µL 1g/L NBD-C6-ceramide, and 1184 µL 0.18 mol/L NaBiEDTA density gradient solution. A volume of 1150 µL of this mixture was then transferred to an 11 x 34 mm thickwall polycarbonate tube and centrifuged in a Beckman Optima MAX-XP centrifuge equipped with a MLA-130 rotor (Beckman Coulter, Inc. Brea, CA) for 6 hr at 4ºC. Following gentle overlayment of hexane, the tubes were immediately imaged by a CCD camera (Quantifire XI, Optronics, Muskogee, OK) with a Fiber-Lite Illuminator (MH100A, Edmund Industrial Optics, Barrington, NJ) as light source. The tube holder, digital camera and illuminator were positioned orthogonally to each other on an optical bench. The respective filters (Semrock, Rochester, NY) were chosen to match NBD excitation (465 nm/60 nm bandwidth, part #

FF01-460-60-25) and emission (>500 nm/long pass, part # BLP01-488R-25) wavelengths, respectively. Pixel values of the center of the tube were converted into fluorescent intensity using Origin 7.5 (OriginLab Ltd, Northampton, MA) software and plotted as a function of the tube coordinate. A total of 10 LP subclasses were identified by their density intervals (131, 134), and quantified by calculation of area under curve (AUC, i.e. pixel value). The major LP subclasses are TRL (d <1.019 g/mL), LDL_1 (d = 1.019-1.023 g/mL), LDL_2 (d = 1.023-1.034 g/mL), LDL_3 (d = 1.034-1.044 g/mL), LDL_4 (d = 1.044-1.063 g/mL), HDL_{2b} (d = 1.063-1.091 g/mL), HDL_{2c} (d = 1.091-1.110 g/mL), HDL_{3a} (d = 1.110-1.133 g/mL), HDL_{3b} (d = 1.133-1.156 g/mL) and HDL_{3c} (d = 1.156-1.179 g/mL).

Methodology was validated by measuring intra- and inter- assay reproducibility of samples differing in TG concentration. Subjects were divided into quintiles based on plasma TG concentration. Three plasma samples from each of the 1st, 3rd, 5th quintiles were randomly selected and pooled and used as low (55.6 mg/dL), medium (102.2 mg/dL) and high TG (199.3 mg/dL) TG concentration samples. For intra-assay variation assessment, 3 replicates for each of the low, medium and high TG samples were analyzed in a single run. For inter-assay variation assessment, 5 replicates of the samples were run on 5 separate days. On each day, a set of low, medium and high sample was analyzed.

Statistics

Statistical analysis was performed using JMP 10.0 (SAS Institute Inc., Cary, North Carolina). All data were assessed for normality using Shapiro-Wilk test. Data with

a skewed distribution were log transformed before subsequent analysis. Baseline characteristics between men and women were compared by unpaired t-test. Measurement of LP subclass by NBD-C6 ceramide distribution was compared with conventional chemical measurements from Roussell (165) using Pearson correlation. The LP density distribution and HOMA-IR at baseline and after each of the 4 test diets were compared by repeated measures ANCOVA with adjustment for age, gender and BMI, followed by Tukey-Kramer test. The primary outcome was the difference in absolute and fractional abundance of small dense LDL_4 after the consumption of BOLD+ diet compared to LDL_4 abundance following the consumption of HAD diet. Pearson correlation coefficients were calculated for the BOLD+ diet induced change in LDL_4 to changes in previously reported lipid variables and apolipoprotein concentrations. To examine the effect of baseline lipids, inflammation indicators and insulin sensitivity on LDL_4 response to test diets, Pearson correlation coefficient was calculated for baseline TG, TC, LDL-C, HDL-C, HOMA-IR and CRP with percentage change of LDL_4 from baseline for each test diet. When a significant correlation was observed for baseline HOMA-IR and LDL_4 percentage change, a secondary analysis was performed to better characterize the effect of baseline HOMA-IR. Subjects with baseline HOMA-IR below the median were classified as low HOMA-IR group (n = 13) and those with HOMA-IR equal or greater than the median were classified as high HOMA-IR group (n = 14). Since there was more individual variation in LDL_4 response observed among subjects with high baseline HOMA-IR, as judged from standard deviation, the median was included in the high HOMA-IR group in the analysis. However, the

inclusion or removal of the median from either the low or high HOMA-IR group had no effect on the significance of any tests. To investigate the responsiveness to test diets within each baseline HOMA-IR subgroup, mixed model repeated measures analysis was performed with subjects entered as a random variable and age, gender and BMI as covariates. When a significant overall dietary effect was detected by the mixed model, Tukey-Kramer test was used to further investigate the difference between diets. The level of significance was $P < 0.05$. Tabular values were expressed as mean ± SEM.

Results

Baseline characteristics

Baseline characteristics of the 27 subjects that had complete sample sets from the original 36 (165) are shown in Table 4.2. The subjects were mildly hypercholesterolemic but were within the normal range for TG (<150 mg/dL) and HDL-C (>40 mg/dL for men and >50 mg/dL for women) concentrations. Females had a significantly higher average total-C ($P = 0.003$) and LDL-C ($P = 0.007$) than males. No difference was observed for TG, HDL-C, CRP and HOMA-IR between men and women. Despite the difference in total LDL-C concentration between men and women, the difference was mainly due to the presence or more large and medium LDL in women (Figure 4.1) but not small dense LDL_4. No significant gender effect on LDL_4 response to the test diets was observed.

LP density distribution

LP density distribution was measured using a slight modification of a isopycnic separation of lipoproteins fluorescently dyed with NBD-C6-ceramide followed by digital analysis (232). NBD-C6-ceramide is only fluorescent under hydrophobic environment.

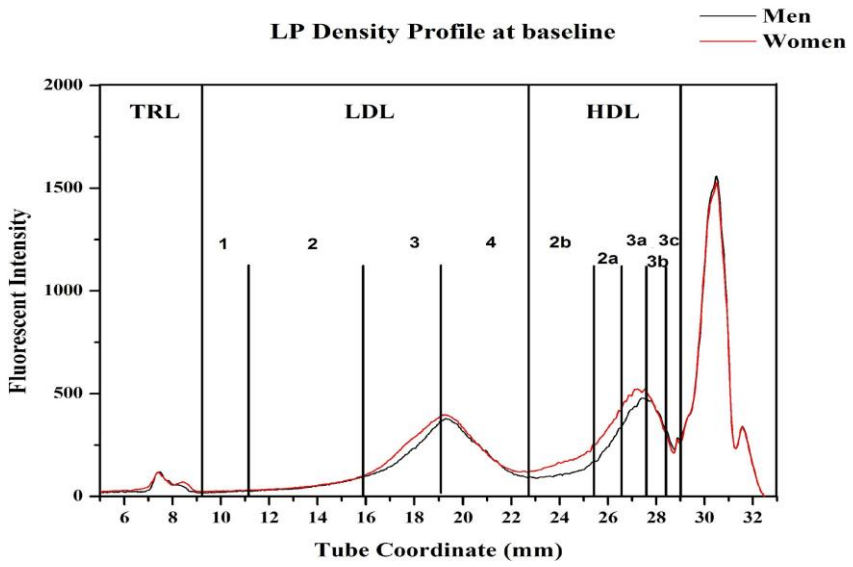
Table 4.2 Baseline characteristics of BOLD Study subjects

	Men (n = 10)	Women (n = 17)	Total (n = 27)
Age (y)	50.9 ± 2.6	50.9 ± 2.2	50.9 ± 1.7
BMI	26.5 ± 0.8	25.1 ± 0.9	25.6 ± 0.6
TG (mg/dL)	95.6 ± 8.9	97.1 ± 7	96.5 ± 5.4
Total-C (mg/dL)	192.3 ± 5.6	225.6 ± 7	213.3 ± 5.8
LDL-C (mg/dL)	125.7 ± 5.2	150.1 ± 5.5	141 ± 4.6
HDL-C (mg/dL)	47.6 ± 2.6	56.1 ± 3.9	52.9 ± 2.7
HOMA-IR	2.8 ± 0.3	2.7 ± 0.2	2.7 ± 0.2
CRP (mg/L)	1.5 ± 0.5	1.2 ± 0.3	1.3 ± 0.3

Data expressed as mean ± SEM.

Thus, the fluorescent intensity of a particle depends on the quantity of the hydrophobic group/molecules on lipoprotein particle surface, allowing an estimation of total mass. The AUC of core-lipid rich TRL, LDL and HDL_2 were strongly correlated with plasma TG, LDL-C and HDL-C, respectively ($r \geq 0.66$, $P < 0.0001$, Figure 4.2A-C). For core lipid-poor HDL_3, there was a significant but weaker association with HDL-C ($r = 0.27$, $P = 0.007$, Figure 4.2D). Since small HDL_3 contains little cholesterol ester *per se*, only a weak, but significant association was observed for the HDL_3 AUC and HDL-C. The LP density distribution demonstrated that technical variations across different LP subclasses were similar for samples containing differing amounts of TG, with the highest variation occurring at the top and bottom of the regions of the tube (i.e. TRL, LDL_1, and HDL_{3c} subclasses). The increase in variability was likely due to the specific solution redistribution condition at these positions during centrifuge deceleration. For other LP subclasses, the average CV% was as low as 5% with 3 replicates for intra-assay assessments and 4% with 5 replicates for inter-assay assessments (Table 4.3).

A.



B.

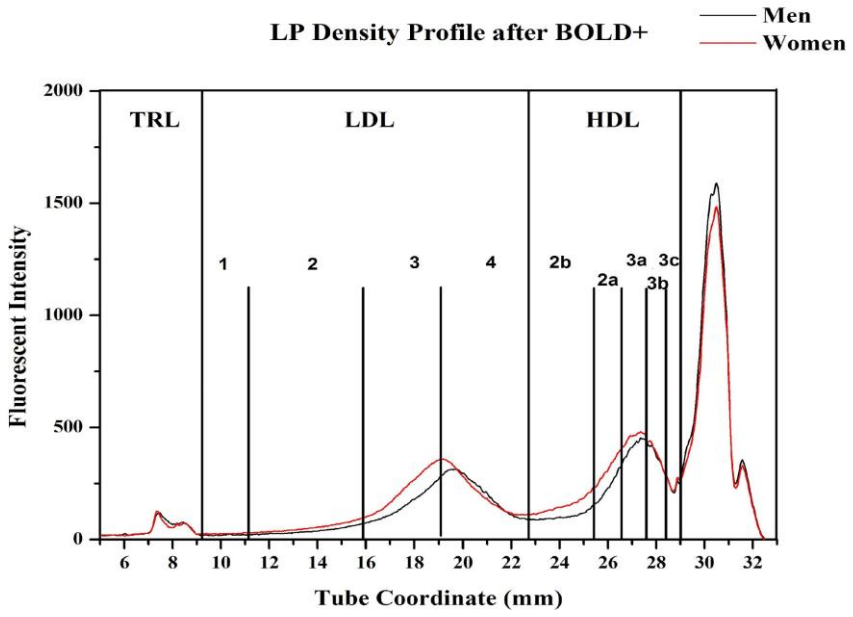


Figure 4.1 LP density profile for male and female subjects at baseline and after the dietary intervention. The BOLD+ diet was used as an example. No significant gender effect on LDL_4 distribution was detected.

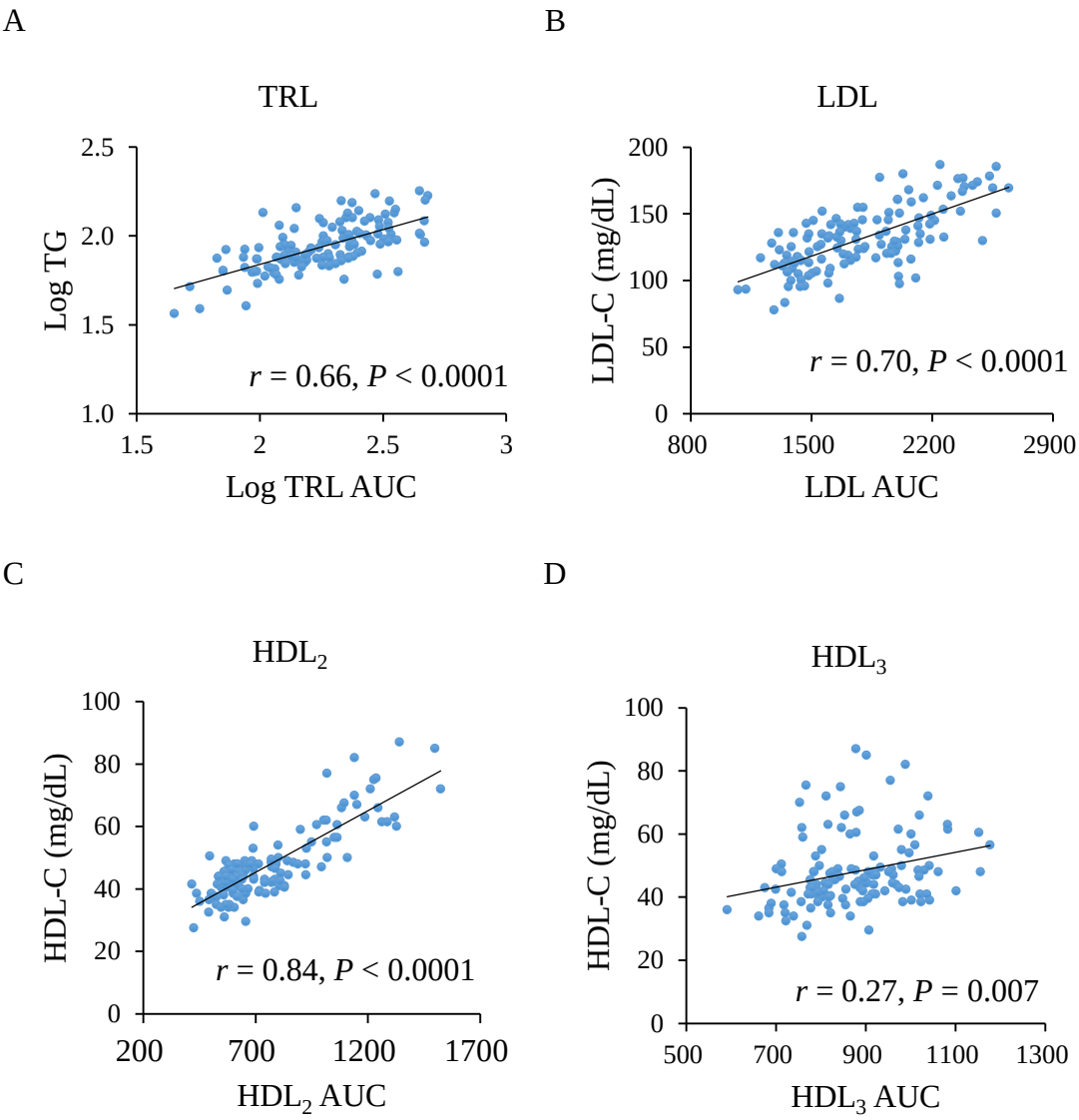


Figure 4.2 Correlations between LP density profile and biochemical measurements in 135 samples. Linear regression, correlation coefficient and *P* value are shown for each comparison. TG and TRL AUC data were log transformed to achieve normality.

Table 4.3 Reproducibility for intra- and inter-assay

	Intra-assay (n = 3)			Inter-assay (n = 5)		
TG	Low	Medium	High	Low	Medium	High
LP distribution calculated as absolute AUC						
TRL	18.6	13.9	14.9	19.8	11.3	10.6
LDL_1	33.6	21.8	12.9	32.6	21.7	8.2
LDL_2	10.1	5.7	8.6	11.3	6.8	4.1
LDL_3	2.4	5.1	8.0	2.9	4.4	4.7
LDL_4	6.2	9.4	8.4	5.4	5.1	7.6
HDL_{2b}	1.5	4.6	6.4	0.2	1.4	2.9
HDL_{2a}	3.4	4.7	6.1	2.0	1.5	0.4
HDL_{3a}	3.2	4.6	4.4	1.9	1.7	1.9
HDL_{3b}	4.3	3.3	1.8	3.0	3.4	6.8
HDL_{3c}	13.9	10.8	9.3	14.4	9.5	13.4
Average	9.7	8.4	8.1	9.3	6.7	6.1
LP distribution calculated as % total AUC						
TRL	10.8	8.8	22.9	9.6	8.8	19.1
LDL_1	21.9	19.2	12.2	21.4	18.1	11.2
LDL_2	5.9	4.3	8.0	4.4	3.4	4.1
LDL_3	5.0	5.6	3.9	5.1	4.9	5.5
LDL_4	4.3	4.3	5.9	3.4	3.5	4.5
HDL_{2b}	3.2	1.9	7.2	2.2	1.9	5.9
HDL_{2a}	8.7	4.2	3.8	7.7	5.0	5.8
HDL_{3a}	6.1	2.3	4.3	5.5	3.4	3.8
HDL_{3b}	4.6	2.0	7.3	4.2	0.8	4.0
HDL_{3c}	11.7	8.4	9.9	9.9	8.2	6.1
Average	8.2	6.1	8.5	7.3	5.8	7.0

Coefficients of variation for intra-assay and inter-assay. Low TG = 55.6 mg/dL, medium TG = 102.2 mg/dL; high TG = 199.3 mg/dL.

Compared with baseline, no absolute or fractional change was observed for any LP subclass after the consumption of the HAD diet (Table 4.4). For this reason, we assumed that macronutrient distribution of the subjects' baseline diet was similar to HAD. Compared with the control HAD diet, BOLD+ significantly reduced total small dense LDL_4 AUC by ~9.2% which also caused a reduction in the fractional abundance of LDL_4 (P <0.05), indicating a shift in LDL particle diameter distribution towards larger sizes. Further analysis demonstrated that when subjects consumed the BOLD+ dietary pattern, the induced decrease in LDL_4 paralleled the decrease in TC (r = 0.48, P = 0.014), LDL-C (r = 0.55, P = 0.004) and ApoB (r = 0.68, P <0.001), with the strongest association observed for ApoB. Despite the lack of a significant difference in TG after the consumption of the HAD and BOLD+ diets, we observed a positive correlation between the change in TG and LDL_4 (r = 0.41, P = 0.044). Total HDL_{2b} AUC was significantly decreased by ~5.6% and ~6.8% after the consumption of the DASH and BOLD diets, respectively (P <0.05), but not after consumption of the BOLD+ diet. The fractional abundance of HDL_{3a} increased following consumption of any of the three test diets low in saturated fat (i.e. DASH, BOLD and BOLD+) compared to the proportion present following consumption of the HAD (P <0.05).

Table 4.4 LP density distribution at baseline and after the test diets

	Baseline	HAD	DASH	BOLD	BOLD+
LP distribution calculated as absolute AUC					
TRL	216 ± 16	204 ± 22	214 ± 14	181 ± 14	195 ± 23
LDL_1	56 ± 5	44 ± 3	47 ± 4	45 ± 4	52 ± 5
LDL_2	274 ± 15^{a}	240 ± 15^{a,b}	232 ± 14^{b}	226 ± 14^{b}	253 ± 21^{a,b}
LDL_3	780 ± 49^{a}	730 ± 47^{a,b}	665 ± 48^{b}	667 ± 48^{b}	687 ± 38^{a,b}
LDL_4	844 ± 39^{a}	822 ± 35^{a}	797 ± 21^{a,b}	761 ± 21^{a,b}	723 ± 33^{b}
HDL_{2b}	369 ± 28^{a}	370 ± 28^{a}	337 ± 22^{b}	331 ± 22^{b}	345 ± 29^{a,b}
HDL_{2a}	438 ± 22	422 ± 24	406 ± 20	408 ± 20	420 ± 26
HDL_{3a}	422 ± 12	395 ± 12	408 ± 12	394 ± 12	399 ± 12
HDL_{3b}	364 ± 9^{a}	343 ± 9^{a,b}	353 ± 8^{b}	329 ± 8^{b}	336 ± 9^{b}
HDL_{3c}	128 ± 5	127 ± 5	129 ± 4	118 ± 4	124 ± 5
LP distribution calculated as % total AUC					
TRL	5.5 ± 0.4	5.6 ± 0.6	5.9 ± 0.4	5.3 ± 0.4	5.6 ± 0.6
LDL_1	1.5 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.5 ± 0.1
LDL_2	7.1 ± 0.3^{a}	6.4 ± 0.3^{a,b}	6.4 ± 0.3^{a,b}	6.5 ± 0.3^{a,b}	7.0 ± 0.3^{a,b}
LDL_3	19.8 ± 0.9	19.4 ± 0.8	18.3 ± 1.0	18.9 ± 1.0	19.1 ± 0.7
LDL_4	21.8 ± 0.8^{a,b}	22.5 ± 0.8^{a}	22.4 ± 0.4^{a}	22.4 ± 0.4^{a}	20.8 ± 0.5^{b}
HDL_{2b}	9.3 ± 0.4	9.9 ± 0.4	9.3 ± 0.3	9.4 ± 0.3	9.5 ± 0.5
HDL_{2a}	11.2 ± 0.2	11.3 ± 0.2	11.3 ± 0.2	11.7 ± 0.2	11.8 ± 0.3
HDL_{3a}	10.9 ± 0.2^{b,c}	10.7 ± 0.2^{c}	11.5 ± 0.2^{a}	11.5 ± 0.2^{a}	11.5 ± 0.3^{a,b}
HDL_{3b}	9.5 ± 0.2	9.4 ± 0.3	9.9 ± 0.2	9.6 ± 0.2	9.7 ± 0.3
HDL_{3c}	3.4 ± 0.1	3.5 ± 0.2	3.6 ± 0.1	3.5 ± 0.1	3.6 ± 0.1

Data were expressed as mean ± SEM. n = 27. LP subclass values with different superscript letters in the same row were significantly different (P <0.05). Skewed data were log transformed before statistical analysis.

HOMA-IR

Average HOMA-IR did not differ from baseline following any of the four test diets (Table 4.2), being 2.76 ± 0.20 following consumption of HAD, 2.64 ± 0.13 following DASH, 2.93 ± 0.17 following BOLD and 2.94 ± 0.17 following BOLD+.

LDL_4 responsiveness

To identify the possible predictors for individual LDL responses to shifts from baseline to HAD or the DASH-like lower SFA diet, simple correlations were calculated for the percentage change in LDL_4 from baseline with baseline lipid variables, HOMA-IR and CRP. The results showed that increased baseline HOMA-IR was strongly associated with a less favorable LDL_4 response to the DASH (r = 0.56, P = 0.003) and BOLD (r = 0.47, P = 0.018) test diets (Table 4.5). Marginally significant correlations were also observed for baseline HOMA-IR (r = 0.36, P = 0.065 and baseline CRP (r = 0.35, P = 0.093) with LDL_4 response to HAD. To better assess the effect of baseline HOMA-IR on the LDL_4 response, subjects were divided into low and high baseline HOMA-IR groups by median split. Subjects with low baseline HOMA-IR had a significant decrease in LDL_4 after the consumption of the BOLD (P = 0.003) and BOLD+ (P = 0.046) diets and a non-significant decrease after the consumption of DASH (P = 0.069) (Figure 4.3). The responsiveness to the four test diets showed no significant difference (P = 0.890) in the low baseline HOMA-IR group. In contrast, subjects with high baseline HOMA-IR showed no change in LDL_4 after consumption of the DASH, BOLD, and BOLD+ diets. The LDL_4 response to the BOLD+ diet was significantly different from the HAD and DASH diets in the high HOMA-IR group (P = 0.013).

Table 4.5 Correlations between LDL_4 change from baseline and baseline lipids, HOMA-IR and CRP for four test diets

	HAD		DASH		BOLD		BOLD+	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
TG	0.16	0.428	0.02	0.922	0.15	0.445	0.16	0.427
TC	0.16	0.412	0.27	0.161	0.13	0.502	0.21	0.300
LDL-C	0.12	0.536	0.22	0.271	0.24	0.222	0.25	0.205
HDL-C	0.21	0.299	0.21	0.288	0.18	0.356	0.05	0.813
HOMA-IR	0.36	0.065	0.56	0.003	0.47	0.018	0.13	0.533
CRP	0.35	0.093	0.23	0.282	0.32	0.125	0.18	0.395

Pearson correlation coefficients. For each intervention diet, n = 27 for lipid variables; n = 25 for HOMA-IR; n = 24 for CRP.

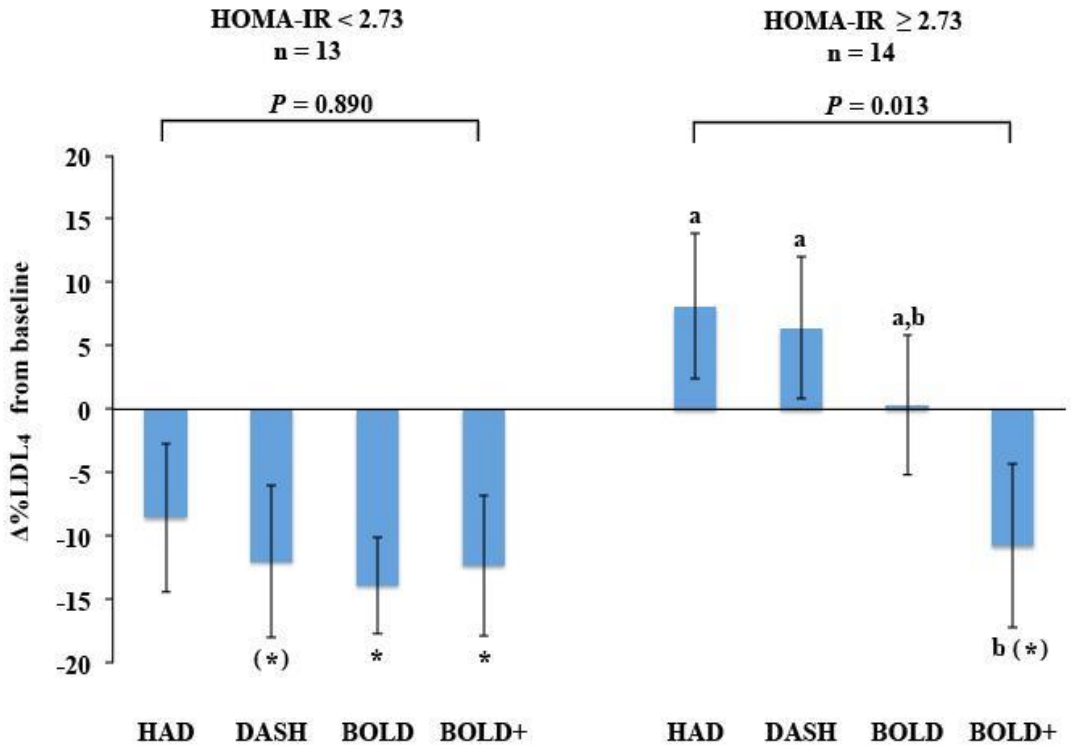


Figure 4.3 Effects of baseline HOMA-IR on LDL_4 response. Low and high baseline HOMA-IR subgroups were classified by median split. Values were expressed as mean ± SEM. Within each subgroup, diet induced LDL_4 percentage changes from baseline were compared by repeated measures ANCOVA with age, gender and BMI adjusted. Different letters indicate significant difference in response to diets (P <0.05). An unbracked asterisk indicates significantly different from zero (P <0.05). A bracketed asterisk indicates marginally different from zero (P <0.1).

Discussion

In the present study, we found that increasing dietary protein, from lean beef and other protein sources, while decreasing carbohydrate in a DASH-like dietary pattern improved LP density profile by decreasing the absolute and proportional abundance of sdLDL (LDL_4) when compared to a HAD. The DASH and BOLD diets also appeared to have beneficial effects on the LDL density profile. However, these effects were modified by baseline HOMA-IR of the subjects. To the best of our knowledge, this is the first study that showed baseline metabolic flexibility modulates the sdLDL response to a low SFA dietary intervention.

Small dense LDL_4 was decreased by 9.6% after consumption of the BOLD+ versus the HAD. It has been previously reported that the BOLD+ diet induced a 6% reduction in LDL-C; the present density distribution analysis identified a preferential decrease in the small and what is thought to be the most atherogenic LDL subfraction. In this analysis, LDL_4 particles were defined as those with a density range of 1.044-1.063 g/mL, which roughly corresponds to LDL particles with a diameter smaller than 24.2 nm measured by non-denaturing gradient gel electrophoresis (233). The large prospective Quebec Cardiovascular Study with over 2,000 men found that participants in the third tertile of absolute or relative cholesterol in LDL particles with diameters smaller than 25.5 nm had over a 4-fold increase in the risk of ischemic heart disease compared with men in the first tertile after adjustments for other lipid and non-lipid risk factors (221). These observations suggest that even a modest reduction in sdLDL concentration may be of significant benefit for CVD risk reduction. Our finding that LDL density distribution

was only changed by the BOLD+ diet, but not the DASH and BOLD diets, suggests that the BOLD+ diet induced improvement in LDL density profile was partially mediated by the replacement of carbohydrates with protein in addition to the effects of SFA reduction. Despite the difference in protein source, this finding is consistent with earlier observations that replacement of carbohydrate by protein in a low SFA diet can improve the LP profile by shifting LDL to larger particle size (225, 227, 234). The underlying mechanism for the decrease in sdLDL by the BOLD+ diet is unclear, but possibly involves alteration in TG metabolism, as shown by our correlation analysis. Higher VLDL ApoB secretion, lower VLDL ApoB fractional transfer rate (FTR), as well as a lower LDL ApoB fractional catabolic rate (FCR) have been observed in healthy men with predominantly small, dense LDL (pattern B)(235).

Our secondary analysis showed that a significant decrease in sdLDL occurred only in subjects with higher baseline metabolic flexibility, as indicated by low HOMA-IR values, after consumption of the DASH and BOLD diets. One earlier study of 86 healthy men, using Step I and Step II diets reported that high baseline HOMA-IR unfavorably affected the change in total LDL cholesterol in those subjects (236). We extended these findings by showing that baseline HOMA-IR also influences the change in LDL density distribution in response to low saturated fat, high carbohydrate diets. This observation may help explain inconsistencies in different studies. In a controlled feeding study of 24 hypercholesterolemic men and women, a Step II diet enriched in whole grains, vegetable and fruits decreased LDL particle size compared with a higher fat “healthy American diet” (237). Since our subjects were younger and had much

milder dyslipidemia, a potential difference in metabolic flexibility may account for the differences in study outcomes. Notably, the subjects in the BOLD study had fasting glucose and insulin concentrations that were within normal limits (165). Interestingly, similar responses to the BOLD+ diet for sdLDL were observed for low and high baseline HOMA-IR groups. Metabolic inflexibility is associated with postprandial hyperglycemia which promotes ApoB glycation and decreases the affinity of ApoB for LDL-R (238); sdLDL is also the subclass most susceptible to glycation (239). Compared with DASH and BOLD diets, BOLD+ had a higher proportion of protein and a lower glycemic load, and thus may attenuate the glycation of LDL, especially in subjects with decreased metabolic flexibility.

Amounts of larger diameter HDL_{2b} were decreased following consumption of the DASH and BOLD dietary patterns when compared to HAD. This observation was consistent with a previous report that the most pronounced HDL response to a low fat and SFA diet is the decrease in large HDL_2 and HDL_{2b} in healthy subjects (240). Turnover studies have showed that low fat diets decrease the transport rate and increase the FCR of ApoA-I while dietary fat has the opposite effect (241, 242). The FCR of ApoA-I is reported to be inversely associated with HDL particle size (243). Caution should be taken in interpreting the decrease in large HDL_{2b} by low fat diets as HDL possess anti-inflammatory and anti-oxidative functions in addition to cholesterol efflux, which have not been fully explored. BOLD+ appeared to modestly reverse the significant decrease in absolute HDL_{2b} concentration caused by the DASH and BOLD dietary patterns, though the magnitude was not large enough to prevent a shift in HDL

towards smaller particles. Apolipoprotein measurements in the original BOLD study showed that HDL ApoC-III was significantly reduced by BOLD+ compared with HAD (165). ApoC-III was found to be preferentially enriched in the large HDL_{2b} subfraction (120) and its enrichment can compromise the anti-inflammatory functionality of HDL (37) and increase risk of CVD (244). A reduction in ApoC-III combined with no change in HDL_{2b} may suggest an improvement in HDL functionality by the BOLD+ dietary pattern.

One limitation of the current study is that it was not designed as a part of the initial BOLD study and had a reduced sample size, which resulted in less statistical power. For main effects, we detected diet-induced changes in LDL_4 and HDL_{2b} as well as total LDL and HDL (data not shown). For the secondary analysis, we failed to detect a significant baseline HOMA-IR and diet interaction ($P = 0.153$) with a mixed model. However, by using the median split, we clearly demonstrated the difference in the LDL_4 response pattern to the test diets in the low and high baseline HOMA-IR groups, which suggests a likely interaction of HOMA-IR with the DASH and BOLD dietary patterns.

In the present study, we showed that the proportion of macronutrient may have a greater influence than protein source on LDL distribution in a heart healthy dietary context. We found that increasing dietary protein at the expense of carbohydrate in a heart healthy diet had favorable effects on LDL distribution. A low saturated fat, moderate protein diet including lean beef improved the LDL density profile by decreasing the absolute and proportional abundance of small dense LDL_4 in a mildly hypercholesterolemic population. We also offer the first evidence to suggest a possible

role for baseline metabolic flexibility in modulating the individual sdLDL response to a low saturated fat dietary pattern, and that partial replacement of carbohydrates by protein in a DASH-like diet may improve health outcomes for individuals with reduced metabolic flexibility. These results combined with previous findings that the BOLD+ dietary pattern was the most effective in decreasing ApoB and systolic blood pressure merit further investigation on the long term effects of DASH-like, moderate protein diets.

CHAPTER V

CONCLUSIONS

We evaluated the health benefits of beef consumption in moderate and low fat dietary context in 2 healthy populations.

In the first study (Chapter II, III), the objective was to evaluate the consumption of moderate fat ground beef consumption on HDL density distribution, composition and functionality. Our primary findings were that, beef consumption improved metabolic flexibility as judged by decreased HOMA-IR score, increased HDL_3 S1P and decreased HDL 5-KETE regardless of ground beef MUFA:SFA ratio. Though we did observe some additional health benefits for high MUFA beef, the effect was mild with no significant endpoint difference between low and high MUFA beef observed. These findings suggest that the improvements in ASCVD risk by ground beef consumption was at least partially mediated by the lean portion of beef, which leads us to the second study.

In the second study (Chapter IV), the objective was to evaluate the effects of lean beef as a main protein source in a DASH-like, heart healthy dietary context on lipoprotein density distribution. The primary finding was that the small dense LDL_4 was only significantly decreased by the BOLD+ diet, which contains the highest proportion of protein (including lean beef) among the 4 intervention diets.

Epidemiologic evidence regarding the impact of red meat consumption on ASCVD remains mixed. Confounders such as sodium and nitrate (processed meat), total

SFA intake and serving size, incorporation in a western dietary pattern may influence risk assessment. In our two randomized cross-over beef intervention studies in two healthy populations, we observed no adverse effects of 4-5 ounce, unprocessed, low-moderate fat beef consumption on ASCVD risk. Importantly, we observed high inter-individual variation in response, especially with advanced lipid assessment. We have demonstrated that individual response to beef intervention is influenced by baseline health status, with more favorable response observed in subject with better metabolic health. Genetic and metabolic typing studies in the future will vastly improve our understanding of the underlying mechanisms.

REFERENCES

1. Gordon DJ, Probstfield JL, Garrison RJ, et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation* 1989;79(1):8-15.

2. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA* 2001;285(19):2486-97.

3. Lavigne PM, Karas RH. The current state of niacin in cardiovascular disease prevention: a systematic review and meta-regression. *J Am Coll Cardiol* 2013;61(4):440-6.

4. Barter PJ, Caulfield M, Eriksson M, et al. Effects of torcetrapib in patients at high risk for coronary events. *N Engl J Med* 2007;357(21):2109-22.

5. Ray KK, Ditmarsch M, Kallend D, et al. The effect of cholesteryl ester transfer protein inhibition on lipids, lipoproteins, and markers of HDL function after an acute coronary syndrome: the dal-ACUTE randomized trial. *Eur Heart J* 2014;35(27):1792-800.

6. Shah AS, Tan L, Long JL, et al. Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond. *J Lipid Res* 2013;54(10):2575-85.

7. Kontush A, Lhomme M, Chapman MJ. Unraveling the complexities of the HDL lipidome. *J Lipid Res* 2013;54(11):2950-63.

8. Marsche G, Saemann MD, Heinemann A, et al. Inflammation alters HDL composition and function: implications for HDL-raising therapies. *Pharmacol Ther* 2013;137(3):341-51.

9. Ross R, Glomset JA. The pathogenesis of atherosclerosis (first of two parts). *N Engl J Med* 1976;295(7):369-77.

10. Van Lenten BJ, Navab M, Shih D, et al. The role of high-density lipoproteins in oxidation and inflammation. *Trends Cardiovasc Med* 2001;11(3-4):155-61.

11. Durrington PN, Mackness B, Mackness MI. Paraoxonase and atherosclerosis. *Arterioscler Thromb Vasc Biol* 2001;21(4):473-80.

12. Tolle M, Pawlak A, Schuchardt M, et al. HDL-associated lysosphingolipids inhibit NAD(P)H oxidase-dependent monocyte chemoattractant protein-1 production. *Arterioscler Thromb Vasc Biol* 2008;28(8):1542-8.

13. Argraves KM, Gazzolo PJ, Groh EM, et al. High density lipoprotein-associated sphingosine-1-phosphate promotes endothelial barrier function. *J Biol Chem* 2008;283(36):25074-81.

14. Zhu X, Lee JY, Timmins JM, et al. Increased cellular free cholesterol in macrophage-specific ABCA1 knock-out mice enhances pro-inflammatory response of macrophages. *J Biol Chem* 2008;283(34):22930-41.

15. De Nardo D, Labzin LI, Kono H, et al. High-density lipoprotein mediates anti-inflammatory reprogramming of macrophages via the transcriptional regulator ATF3. *Nat Immunol* 2014;15(2):152-60.

16. Nofer JR, van der Giet M, Tolle M, et al. HDL induces NO-dependent vasorelaxation via the lysophospholipid receptor $S1P_3$. *J Clin Invest* 2004;113(4):569-81.

17. Yuhanna IS, Zhu Y, Cox BE, et al. High-density lipoprotein binding to scavenger receptor-BI activates endothelial nitric oxide synthase. *Nat Med* 2001;7(7):853-7.

18. Nofer JR, Levkau B, Wolinska I, et al. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J Biol Chem* 2001;276(37):34480-5.

19. Eggerman TL, Hoeg JM, Meng MS, et al. Differential tissue-specific expression of human apoA-I and apoA-II. *J Lipid Res* 1991;32(5):821-8.

20. Timmins JM, Lee JY, Boudyguina E, et al. Targeted inactivation of hepatic ABCA1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. *J Clin Invest* 2005;115(5):1333-42.

21. Brunham LR, Kruit JK, Iqbal J, et al. Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. *J Clin Invest* 2006;116(4):1052-62.

22. Murdoch SJ, Breckenridge WC. Influence of lipoprotein lipase and hepatic lipase on the transformation of VLDL and HDL during lipolysis of VLDL. *Atherosclerosis* 1995;118(2):193-212.

23. Santamarina-Fojo S, Lambert G, Hoeg JM, et al. Lecithin-cholesterol acyltransferase: role in lipoprotein metabolism, reverse cholesterol transport and atherosclerosis. *Curr Opin Lipidol* 2000;11(3):267-75.

24. Kennedy MA, Barrera GC, Nakamura K, et al. ABCG1 has a critical role in mediating cholesterol efflux to HDL and preventing cellular lipid accumulation. *Cell Metab* 2005;1(2):121-31.

25. Yancey PG, de la Llera-Moya M, Swarnakar S, et al. High density lipoprotein phospholipid composition is a major determinant of the bi-directional flux and net movement of cellular free cholesterol mediated by scavenger receptor BI. *J Biol Chem* 2000;275(47):36596-604.

26. Tall AR. Plasma cholesteryl ester transfer protein. *J Lipid Res* 1993;34(8):1255-74.

27. Tall AR, Krumholz S, Olivercrona T, et al. Plasma phospholipid transfer protein enhances transfer and exchange of phospholipids between very low-density lipoproteins and high-density lipoproteins during lipolysis. *J Lipid Res* 1985;26(7):842-51.

28. Jauhiainen M, Metso J, Pahlman R, et al. Human plasma phospholipid transfer protein causes high density lipoprotein conversion. *J Biol Chem* 1993;268(6):4032-6.

29. Barter PJ. Hugh sinclair lecture: the regulation and remodelling of HDL by plasma factors. *Atheroscler Suppl* 2002;3(4):39-47.

30. Acton S, Rigotti A, Landschulz KT, et al. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 1996;271(5248):518-20.

31. Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ Res* 2005;96(12):1221-32.

32. Rached FH, Chapman MJ, Kontush A. HDL particle subpopulations: focus on biological function. *Biofactors* 2015;41(2):67-77.

33. Gordon SM, Davidson WS, Urbina EM, et al. The effects of type 2 diabetes on lipoprotein composition and arterial stiffness in male youth. *Diabetes* 2013;62(8):2958-67.

34. Frank PG, Marcel YL. Apolipoprotein A-I: structure-function relationships. *J Lipid Res* 2000;41(6):853-72.

35. Davidson WS, Thompson TB. The structure of apolipoprotein A-I in high density lipoproteins. *J Biol Chem* 2007;282(31):22249-53.

36. Di Angelantonio E, Sarwar N, Kaptoge S, et al. Major lipids, apolipoproteins, and risk of vascular disease. *JAMA* 2009;302(18):1993-2000.

37. Rothblat GH, Phillips MC. High-density lipoprotein heterogeneity and function in reverse cholesterol transport. *Curr Opin Lipidol* 2010;21(3):229-38.

38. Navab M, Hama SY, Cooke CJ, et al. Normal high density lipoprotein inhibits three steps in the formation of mildly oxidized low density lipoprotein: step 1. *J Lipid Res* 2000;41(9):1481-94.

39. Garner B, Waldeck AR, Witting PK, et al. Oxidation of high density lipoproteins. II. Evidence for direct reduction of lipid hydroperoxides by methionine residues of apolipoproteins AI and AII. *J Biol Chem* 1998;273(11):6088-95.

40. Sorenson RC, Bisgaier CL, Aviram M, et al. Human serum Paraoxonase/Arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids: apolipoprotein A-I stabilizes activity. *Arterioscler Thromb Vasc Biol* 1999;19(9):2214-25.

41. Patel S, Drew BG, Nakhla S, et al. Reconstituted high-density lipoprotein increases plasma high-density lipoprotein anti-inflammatory properties and cholesterol efflux capacity in patients with type 2 diabetes. *J Am Coll Cardiol* 2009;53(11):962-71.

42. Nicholls SJ, Dusting GJ, Cutri B, et al. Reconstituted high-density lipoproteins inhibit the acute pro-oxidant and proinflammatory vascular changes induced by a periarterial collar in normocholesterolemic rabbits. *Circulation* 2005;111(12):1543-50.

43. Li Y, Dong JB, Wu MP. Human ApoA-I overexpression diminishes LPS-induced systemic inflammation and multiple organ damage in mice. *Eur J Pharmacol* 2008;590(1-3):417-22.

44. de Souza JA, Vindis C, Negre-Salvayre A, et al. Small, dense HDL_3 particles attenuate apoptosis in endothelial cells: pivotal role of apolipoprotein A-I. *J Cell Mol Med* 2010;14(3):608-20.

45. Escola-Gil JC, Blanco-Vaca E, Julve J. Overexpression of human apolipoprotein A-II in transgenic mice does not increase their susceptibility to insulin resistance and obesity. *Diabetologia* 2002;45(4):600-1.

46. Castellani LW, Navab M, Van Lenten BJ, et al. Overexpression of apolipoprotein AII in transgenic mice converts high density lipoproteins to proinflammatory particles. *J Clin Invest* 1997;100(2):464-74.

47. Boisfer E, Stengel D, Pastier D, et al. Antioxidant properties of HDL in transgenic mice overexpressing human apolipoprotein A-II. *J Lipid Res* 2002;43(5):732-41.

48. Luc G, Bard JM, Ferrieres J, et al. Value of HDL cholesterol, apolipoprotein A-I, lipoprotein A-I, and lipoprotein A-I/A-II in prediction of coronary heart disease: the PRIME study. *Arterioscler Thromb Vasc Biol* 2002;22(7):1155-61.

49. Fager G, Wiklund O, Olofsson SO, et al. Multivariate analyses of serum apolipoproteins and risk factors in relation to acute myocardial infarction. *Arteriosclerosis* 1981;1(4):273-9.

50. Buring JE, O'Connor GT, Goldhaber SZ, et al. Decreased HDL_2 and HDL_3 cholesterol, ApoA-I and ApoA-II, and increased risk of myocardial infarction. *Circulation* 1992;85(1):22-9.

51. Genest JJ, Jr., Bard JM, Fruchart JC, et al. Plasma apolipoprotein A-I, A-II, B, E and C-III containing particles in men with premature coronary artery disease. *Atherosclerosis* 1991;90(2-3):149-57.

52. Brousseau T, Dupuy-Gorce AM, Evans A, et al. Significant impact of the highly informative (CA)n repeat polymorphism of the ApoA-II gene on the plasma APOA-II concentrations and HDL subfractions: the ECTIM study. *Am J Med Genet* 2002;110(1):19-24.

53. Weisgraber KH, Mahley RW. Subfractionation of human high density lipoproteins by heparin-Sepharose affinity chromatography. *J Lipid Res* 1980;21(3):316-25.

54. Wilson C, Wardell MR, Weisgraber KH, et al. Three-dimensional structure of the LDL receptor-binding domain of human apolipoprotein E. *Science* 1991;252(5014):1817-22.

55. Vedhachalam C, Narayanaswami V, Neto N, et al. The C-terminal lipid-binding domain of apolipoprotein E is a highly efficient mediator of ABCA1-dependent cholesterol efflux that promotes the assembly of high-density lipoproteins. *Biochemistry* 2007;46(10):2583-93.

56. Koo C, Innerarity TL, Mahley RW. Obligatory role of cholesterol and apolipoprotein E in the formation of large cholesterol-enriched and receptor-active high density lipoproteins. *J Biol Chem* 1985;260(22):11934-43.

57. Matsuura F, Wang N, Chen WG, et al. HDL from CETP-deficient subjects shows enhanced ability to promote cholesterol efflux from macrophages in an apoE- and ABCG1-dependent pathway. *J Clin Invest* 2006;116(5):1435-42.

58. Mahley RW. Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science* 1988;240(4852):622-30.

59. Chroni A, Nieland TJ, Kypreos KE, et al. SR-BI mediates cholesterol efflux via its interactions with lipid-bound ApoE. Structural mutations in SR-BI diminish cholesterol efflux. *Biochemistry* 2005;44(39):13132-43.

60. Kothapalli D, Liu SL, Bae YH, et al. Cardiovascular protection by ApoE and ApoE-HDL linked to suppression of ECM gene expression and arterial stiffening. *Cell Rep* 2012;2(5):1259-71.

61. Murata N, Sato K, Kon J, et al. Interaction of sphingosine-1-phosphate with plasma components, including lipoproteins, regulates the lipid receptor-mediated actions. *Biochem J* 2000;352 Pt 3:809-15.

62. Christoffersen C, Obinata H, Kumaraswamy SB, et al. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. *Proc Natl Acad Sci USA* 2011;108(23):9613-8.

63. Yatomi Y. Sphingosine-1-phosphate in vascular biology: possible therapeutic strategies to control vascular diseases. *Curr Pharm Des* 2006;12(5):575-87.

64. Hanel P, Andreani P, Graler MH. Erythrocytes store and release sphingosine-1-phosphate in blood. *Faseb J* 2007;21(4):1202-9.

65. Yatomi Y, Igarashi Y, Yang LB, et al. Sphingosine-1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J Biochem* 1997;121(5):969-73.

66. Wilkerson BA, Grass GD, Wing SB, et al. Sphingosine-1-phosphate (S1P) carrier-dependent regulation of endothelial barrier: high density lipoprotein (HDL)-S1P prolongs endothelial barrier enhancement as compared with albumin-S1P via effects on levels, trafficking, and signaling of $S1P_1$. *J Biol Chem* 2012;287(53):44645-53.

67. Kimura T, Sato K, Kuwabara A, et al. Sphingosine-1-phosphate may be a major component of plasma lipoproteins responsible for the cytoprotective actions in human umbilical vein endothelial cells. *J Biol Chem* 2001;276(34):31780-5.

68. Kimura T, Sato K, Malchinkhuu E, et al. High-density lipoprotein stimulates endothelial cell migration and survival through sphingosine-1-phosphate and its receptors. *Arterioscler Thromb Vasc Biol* 2003;23(7):1283-8.

69. Kimura T, Tomura H, Mogi C, et al. Role of scavenger receptor class B type I and sphingosine-1-phosphate receptors in high density lipoprotein-induced inhibition of adhesion molecule expression in endothelial cells. *J Biol Chem* 2006;281(49):37457-67.

70. Norata GD, Marchesi P, Pirillo A, et al. Long pentraxin 3, a key component of innate immunity, is modulated by high-density lipoproteins in endothelial cells. *Arterioscler Thromb Vasc Biol* 2008;28(5):925-31.

71. Miller AV, Alvarez SE, Spiegel S, et al. Sphingosine kinases and sphingosine-1-phosphate are critical for transforming growth factor beta-induced extracellular signal-regulated kinase 1 and 2 activation and promotion of migration and invasion of esophageal cancer cells. *Mol Cell Biol* 2008;28(12):4142-51.

72. Sattler KJ, Elbasan S, Keul P, et al. Sphingosine-1-phosphate levels in plasma and HDL are altered in coronary artery disease. *Basic Res Cardiol* 2010;105(6):821-32.

73. Argraves KM, Sethi AA, Gazzolo PJ, et al. S1P, dihydro-S1P and C24:1-ceramide levels in the HDL-containing fraction of serum inversely correlate with occurrence of ischemic heart disease. *Lipids Health Dis* 2011;10:70.

74. Sattler K, Lehmann I, Graler M, et al. HDL-bound sphingosine-1-phosphate (S1P) predicts the severity of coronary artery atherosclerosis. *Cell Physiol Biochem* 2014;34(1):172-84.

75. Jing XD, Wei XM, Deng SB, et al. The relationship between the high-density lipoprotein (HDL)-associated sphingosine-1-phosphate (S1P) and coronary in-stent restenosis. *Clin Chim Acta* 2015;446:248-52.

76. Tourdot BE, Ahmed I, Holinstat M. The emerging role of oxylipins in thrombosis and diabetes. *Front Pharmacol* 2014;4:176.

77. Strassburg K, Huijbrechts AM, Kortekaas KA, et al. Quantitative profiling of oxylipins through comprehensive LC-MS/MS analysis: application in cardiac surgery. *Anal Bioanal Chem* 2012;404(5):1413-26.

78. Shearer GC, Newman JW. Lipoprotein lipase releases esterified oxylipins from very low-density lipoproteins. *Prostaglandins Leukot Essent Fatty Acids* 2008;79(6):215-22.

79. Zivkovic A, Yang J, Georgi K, et al. Serum oxylipin profiles in IgA nephropathy patients reflect kidney functional alterations. *Metabolomics* 2012;8(6):1102-13.

80. Hammond VJ, O'Donnell VB. Esterified eicosanoids: generation, characterization and function. *Biochim Biophys Acta* 2012;1818(10):2403-12.

81. Niki E. Lipid peroxidation products as oxidative stress biomarkers. *BioFactors* 2008;34(2):171-80.

82. Poeckel D, Funk CD. The 5-lipoxygenase/leukotriene pathway in preclinical models of cardiovascular disease. *Cardiovasc Res* 2010;86(2):243-53.

83. Shearer GC, Harris WS, Pedersen TL, et al. Detection of omega-3 oxylipins in human plasma and response to treatment with omega-3 acid ethyl esters. *J Lipid Res* 2010;51(8):2074-81.

84. Kuhn H, O'Donnell VB. Inflammation and immune regulation by 12/15-lipoxygenases. *Prog Lipid Res* 2006;45(4):334-56.

85. Imig JD. Epoxides and soluble epoxide hydrolase in cardiovascular physiology. *Physiol Rev* 2012;92(1):101-30.

86. O'Donnell VB, Murphy RC. New families of bioactive oxidized phospholipids generated by immune cells: identification and signaling actions. *Blood* 2012;120(10):1985-92.

87. Mulugeta S, Suzuki T, Hernandez NT, et al. Identification and absolute configuration of dihydroxy-arachidonic acids formed by oxygenation of 5S-HETE by native and aspirin-acetylated COX-2. *J Lipid Res* 2010;51(3):575-85.

88. Schneider C, Yu Z, Boeglin WE, et al. Enantiomeric separation of hydroxy and hydroperoxy eicosanoids by chiral column chromatography. *Methods Enzymol* 2007;433:145-57.

89. Bylund J, Kunz T, Valmsen K, et al. Cytochromes P450 with bisallylic hydroxylation activity on arachidonic and linoleic acids studied with human recombinant enzymes and with human and rat liver microsomes. *J Pharmacol Exp Ther* 1998;284(1):51-60.

90. Newman JW, Kaysen GA, Hammock BD, et al. Proteinuria increases oxylipid concentrations in VLDL and HDL but not LDL particles in the rat. *J Lipid Res* 2007;48(8):1792-800.

91. Barlic J, Murphy PM. An oxidized lipid-peroxisome proliferator-activated receptor gamma-chemokine pathway in the regulation of macrophage-vascular smooth muscle cell adhesion. *Trends Cardiovasc Med* 2007;17(8):269-74.

92. Wenk MR. The emerging field of lipidomics. *Nature Rev Drug Discov* 2005;4(7):594-610.

93. Grapov D, Adams SH, Pedersen TL, et al. Type 2 diabetes associated changes in the plasma non-esterified fatty acids, oxylipins and endocannabinoids. *PloS One* 2012;7(11):e48852.

94. Gordon T, Castelli WP, Hjortland MC, et al. High-density lipoprotein as a protective factor against coronary heart disease: the Framingham study. *Am J Med* 1977;62(5):707-14.

95. Van Lenten BJ, Hama SY, de Beer FC, et al. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest* 1995;96(6):2758-67.

96. Ansell BJ, Navab M, Hama S, et al. Inflammatory/antiinflammatory properties of high-density lipoprotein distinguish patients from control subjects better than high-density lipoprotein cholesterol levels and are favorably affected by simvastatin treatment. *Circulation* 2003;108(22):2751-6.

97. Sorrentino SA, Besler C, Rohrer L, et al. Endothelial-vasoprotective effects of high-density lipoprotein are impaired in patients with type 2 diabetes mellitus but are improved after extended-release niacin therapy. *Circulation* 2010;121(1):110-22.

98. Besler C, Heinrich K, Rohrer L, et al. Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. *J Clin Invest* 2011;121(7):2693-708.

99. Riwanto M, Rohrer L, Roschitzki B, et al. Altered activation of endothelial anti- and proapoptotic pathways by high-density lipoprotein from patients with coronary artery disease: role of high-density lipoprotein-proteome remodeling. *Circulation* 2013;127(8):891-904.

100. Nobecourt E, Jacqueminet S, Hansel B, et al. Defective antioxidative activity of small dense HDL_3 particles in type 2 diabetes: relationship to elevated oxidative stress and hyperglycaemia. *Diabetologia* 2005;48(3):529-38.

101. Kontush A, de Faria EC, Chantepie S, et al. A normotriglyceridemic, low HDL-cholesterol phenotype is characterised by elevated oxidative stress and HDL particles with attenuated antioxidative activity. *Atherosclerosis* 2005;182(2):277-85.

102. Banka CL, Yuan T, Debeer MC, et al. Serum Amyloid-A (SAA): influence on HDL-mediated cellular cholesterol efflux. *J Lipid Res* 1995;36(5):1058-65.

103. Brites FD, Bonavita CD, De Geitere C, et al. Alterations in the main steps of reverse cholesterol transport in male patients with primary hypertriglyceridemia and low HDL-cholesterol levels. *Atherosclerosis* 2000;152(1):181-92.

104. Bergt C, Pennathur S, Fu XY, et al. The myeloperoxidase product hypochlorous acid oxidizes HDL in the human artery wall and impairs ABCA1-dependent cholesterol transport. *Proc Natl Acad Sci USA* 2004;101(35):13032-7.

105. Gijon MA, Leslie CC. Regulation of arachidonic acid release and cytosolic phospholipase A2 activation. *J Leukoc Biol* 1999;65(3):330-6.

106. Radmark O, Samuelsson B. Regulation of 5-lipoxygenase enzyme activity. *Biochem Bioph Res Commun* 2005;338(1):102-10.

107. Morgantini C, Meriwether D, Baldi S, et al. HDL lipid composition is profoundly altered in patients with type 2 diabetes and atherosclerotic vascular disease. *Nutr Metab Cardiovas* 2014;24(6):594-9.

108. Kim JB, Hama S, Hough G, et al. Heart failure is associated with impaired anti-inflammatory and antioxidant properties of high-density lipoproteins. *Am J Cardiol* 2013;112(11):1770-7.

109. Carr AC, McCall MR, Frei B. Oxidation of LDL by myeloperoxidase and reactive nitrogen species - Reaction pathways and antioxidant protection. *Arterioscler Thromb Vasc Biol* 2000;20(7):1716-23.

110. Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, et al. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc Natl Acad Sci USA* 1990;87(18):6959-63.

111. Shao BH, Oda MN, Bergt C, et al. Myeloperoxidase impairs ABCA1-dependent cholesterol efflux through methionine oxidation and site-specific tyrosine chlorination of apolipoprotein A-I. *J Biol Chem* 2006;281(14):9001-4.

112. Peng DQ, Brubaker G, Wu Z, et al. Apolipoprotein A-I tryptophan substitution leads to resistance to myeloperoxidase-mediated loss of function. *Arterioscler Thromb Vasc Biol* 2008;28(11):2063-70.

113. Shao B, Tang C, Sinha A, et al. Humans with atherosclerosis have impaired ABCA1 cholesterol efflux and enhanced high-density lipoprotein oxidation by myeloperoxidase. *Circ Res* 2014;114(11):1733-42.

114. Fisher EA, Feig JE, Hewing B, et al. High-density lipoprotein function, dysfunction, and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol* 2012;32(12):2813-20.

115. Holzer M, Zangger K, El-Gamal D, et al. Myeloperoxidase-derived chlorinating species induce protein carbamylation through decomposition of thiocyanate and urea: novel pathways generating dysfunctional high-density lipoprotein. *Antioxid Redox Sign* 2012;17(8):1043-52.

116. Shao B, Cavigiolio G, Brot N, et al. Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I. *Proc Natl Acad Sci USA* 2008;105(34):12224-9.

117. Marsche G, Heller R, Fauler G, et al. 2-chlorohexadecanal derived from hypochlorite-modified high-density lipoprotein-associated plasmalogen is a natural inhibitor of endothelial nitric oxide biosynthesis. *Arterioscler Thromb Vasc Biol* 2004;24(12):2302-6.

118. Pirillo A, Uboldi P, Kuhn H, et al. 15-Lipoxygenase-mediated modification of high density lipoproteins impairs SR-BI- and ABCA1-dependent cholesterol efflux from macrophages. *Biochim Biophys Acta* 2006;1761:292-300.

119. van der Westhuyzen DR, de Beer FC, Webb NR. HDL cholesterol transport during inflammation. *Curr Opin Lipidol* 2007;18(2):147-51.

120. Davidson WS, Silva RA, Chantepie S, et al. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler Thromb Vasc Biol* 2009;29(6):870-6.

121. Kontush A, Therond P, Zerrad A, et al. Preferential sphingosine-1-phosphate enrichment and sphingomyelin depletion are key features of small dense HDL_3

particles: relevance to antiapoptotic and antioxidative activities. *Arterioscler Thromb Vasc Biol* 2007;27(8):1843-9.

122. de Beer MC, Ji AL, Jahangiri A, et al. ATP binding cassette G1-dependent cholesterol efflux during inflammation. *J Lipid Res* 2011;52(2):345-53.

123. Shridas P, Bailey WM, Gizard F, et al. Group X secretory phospholipase A2 negatively regulates ABCA1 and ABCG1 expression and cholesterol efflux in macrophages. *Arterioscler Thromb Vasc Biol* 2010;30(10):2014-21.

124. Khovidhunkit W, Moser AH, Shigenaga JK, et al. Regulation of scavenger receptor class B type I in hamster liver and Hep3B cells by endotoxin and cytokines. *J Lipid Res* 2001;42(10):1636-44.

125. Zeljkovic A, Vekic J, Spasojevic-Kalimanovska V, et al. LDL and HDL subclasses in acute ischemic stroke: prediction of risk and short-term mortality. *Atherosclerosis* 2010;210(2):548-54.

126. Arsenault BJ, Lemieux I, Despres JP, et al. HDL particle size and the risk of coronary heart disease in apparently healthy men and women: the EPIC-Norfolk prospective population study. *Atherosclerosis* 2009;206(1):276-81.

127. Watanabe H, Soderlund S, Soro-Paavonen A, et al. Decreased high-density lipoprotein (HDL) particle size, prebeta-, and large HDL subspecies concentration in Finnish low-HDL families: relationship with intima-media thickness. *Arterioscler Thromb Vasc Biol* 2006;26(4):897-902.

128. Yang YY, Yan BY, Fu MD, et al. Relationship between plasma lipid concentrations and HDL subclasses. *Clin Chim Acta* 2005;354(1-2):49-58.

129. Xu YH, Fu MD. Alterations of HDL subclasses in hyperlipidemia. *Clin Chim Acta* 2003;332(1-2):95-102.

130. Sasahara T, Yamashita T, Sviridov D, et al. Altered properties of high density lipoprotein subfractions in obese subjects. *J Lipid Res* 1997;38(3):600-11.

131. Rosenson RS, Brewer HB, Jr., Chapman MJ, et al. HDL measures, particle heterogeneity, proposed nomenclature, and relation to atherosclerotic cardiovascular events. *Clin Chem* 2011;57(3):392-410.

132. Birner-Gruenberger R, Schittmayer M, Holzer M, et al. Understanding high-density lipoprotein function in disease: recent advances in proteomics unravel the complexity of its composition and biology. *Prog Lipid Res* 2014;56:36-46.

133. Gofman JW, Young W, Tandy R. Ischemic heart disease, atherosclerosis, and longevity. *Circulation* 1966;34(4):679-97.

134. Chapman MJ, Goldstein S, Lagrange D, et al. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res* 1981;22(2):339-58.

135. Kulkarni KR, Marcovina SM, Krauss RM, et al. Quantification of HDL_2 and HDL_3 cholesterol by the Vertical Auto Profile-II (VAP-II) methodology. *J Lipid Res* 1997;38(11):2353-64.

136. Superko HR. Advanced lipoprotein testing and subfractionation are clinically useful. *Circulation* 2009;119(17):2383-95.

137. Kunitake ST, Kane JP. Factors affecting the integrity of high density lipoproteins in the ultracentrifuge. *J Lipid Res* 1982;23(6):936-40.

138. Larner CD, Henriquez RR, Johnson JD, et al. Developing high performance lipoprotein density profiling for use in clinical studies relating to cardiovascular disease. *Anal Chem* 2011;83(22):8524-30.

139. Johnson JD, Bell NJ, Donahoe EL, et al. Metal ion complexes of EDTA as solutes for density gradient ultracentrifugation: influence of metal ions. *Anal Chem* 2005;77(21):7054-61.

140. Hu FB, Manson JE, Willett WC. Types of dietary fat and risk of coronary heart disease: a critical review. *J Am Coll Nutr* 2001;20(1):5-19.

141. Nicholls SJ, Lundman P, Harmer JA, et al. Consumption of saturated fat impairs the anti-inflammatory properties of high-density lipoproteins and endothelial function. *J Am Coll Cardiol* 2006;48(4):715-20.

142. Keogh JB, Grieger JA, Noakes M, et al. Flow-mediated dilatation is impaired by a high-saturated fat diet but not by a high-carbohydrate diet. *Arterioscler Thromb Vasc Biol* 2005;25(6):1274-9.

143. Kennedy A, Martinez K, Chuang CC, et al. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. *J Nutr* 2009;139(1):1-4.

144. Krauss RM, Eckel RH, Howard B, et al. AHA Dietary Guidelines: revision 2000: a statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Stroke* 2000;31(11):2751-66.

145. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Third report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). Final report. *Circulation* 2002;106(25):3143-421.

146. Feinleib M. Seven countries: a multivariate analysis of death and coronary heart disease. *JAMA* 1981;245(5):511-2.

147. Kris-Etherton PM, Pearson TA, Wan Y, et al. High-monounsaturated fatty acid diets lower both plasma cholesterol and triacylglycerol concentrations. *Am J Clin Nutr* 1999;70(6):1009-15.

148. Mensink RP, Zock PL, Kester AD, et al. Effects of dietary fatty acids and carbohydrates on the ratio of serum total to HDL cholesterol and on serum lipids and apolipoproteins: a meta-analysis of 60 controlled trials. *Am J Clin Nutr* 2003;77(5):1146-55.

149. van Dijk SJ, Feskens EJ, Bos MB, et al. A saturated fatty acid-rich diet induces an obesity-linked proinflammatory gene expression profile in adipose tissue of subjects at risk of metabolic syndrome. *Am J Clin Nutr* 2009;90(6):1656-64.

150. Rasmussen BM, Vessby B, Uusitupa M, et al. Effects of dietary saturated, monounsaturated, and n-3 fatty acids on blood pressure in healthy subjects. *Am J Clin Nutr* 2006;83(2):221-6.

151. Guenther PM, Jensen HH, Batres-Marquez SP, et al. Sociodemographic, knowledge, and attitudinal factors related to meat consumption in the United States. *J Am Diet Assoc* 2005;105(8):1266-74.

152. Zanovec M, O'Neil CE, Keast DR, et al. Lean beef contributes significant amounts of key nutrients to the diets of US adults: National Health and Nutrition Examination Survey 1999-2004. *Nutr Res* 2010;30(6):375-81.

153. Cotton PA, Subar AF, Friday JE, et al. Dietary sources of nutrients among US adults, 1994 to 1996. *J Am Diet Assoc* 2004;104(6):921-30.

154. Subar AF, Krebs-Smith SM, Cook A, et al. Dietary sources of nutrients among US adults, 1989 to 1991. *J Am Diet Assoc* 1998;98(5):537-47.

155. Smith SB, Lunt DK, Chung KY, et al. Adiposity, fatty acid composition, and delta-9 desaturase activity during growth in beef cattle. *Anim Sci J* 2006;77(5):478-86.

156. Chung KY, Lunt DK, Kawachi H, et al. Lipogenesis and stearoyl-CoA desaturase gene expression and enzyme activity in adipose tissue of short- and long-fed Angus and Wagyu steers fed corn- or hay-based diets. *J Anim Sci* 2007;85(2):380-7.

157. Alfaia CPM, Alves SP, Martins SIV, et al. Effect of the feeding system on intramuscular fatty acids and conjugated linoleic acid isomers of beef cattle, with emphasis on their nutritional value and discriminatory ability. *Food Chem* 2009;114(3):939-46.

158. Ponnampalam EN, Mann NJ, Sinclair AJ. Effect of feeding systems on omega-3 fatty acids, conjugated linoleic acid and trans fatty acids in Australian beef cuts: potential impact on human health. *Asia Pac J Clin Nutr* 2006;15(1):21-9.

159. Davis CG, Lin B-H. Factors affecting US beef consumption. Washington, DC: Electronic outlook report from ERS, LDP-M-135-02, October 2005.

160. Adams TH, Walzem RL, Smith DR, et al. Hamburger high in total, saturated and trans-fatty acids decreases HDL cholesterol and LDL particle diameter, and increases TAG, in mildly hypercholesterolaemic men. *Brit J Nutr* 2010;103(1):91-8.

161. Richter CK, Skulas-Ray AC, Champagne CM, et al. Plant protein and animal proteins: do they differentially affect cardiovascular disease risk? *Adv Nutr* 2015;6(6):712-28.

162. Gilmore LA, Walzem RL, Crouse SF, et al. Consumption of high-oleic acid ground beef increases HDL-cholesterol concentration but both high- and low-oleic acid ground beef decrease HDL particle diameter in normocholesterolemic men. *J Nutr* 2011;141(6):1188-94.

163. Conlin PR, Chow D, Miller ER, et al. The effect of dietary patterns on blood pressure control in hypertensive patients: results from the Dietary Approaches to Stop Hypertension (DASH) trial. *Am J Hypertens* 2000;13(9):949-55.

164. Appel LJ, Moore TJ, Obarzanek E, et al. A clinical trial of the effects of dietary patterns on blood pressure. *N Engl J Med* 1997;336(16):1117-24.

165. Roussell MA, Hill AM, Gaugler TL, et al. Beef in an Optimal Lean Diet study: effects on lipids, lipoproteins, and apolipoproteins. *Am J Clin Nutr* 2012;95(1):9-16.

166. Hafiane A, Genest J. HDL, atherosclerosis, and emerging therapies. *Cholesterol* 2013;2013:891403.

167. Heinecke JW. The HDL proteome: a marker-and perhaps mediator-of coronary artery disease. *J Lipid Res* 2009;50:S167-71.

168. Freedman DS, Otvos JD, Jeyarajah EJ, et al. Relation of lipoprotein subclasses as measured by proton nuclear magnetic resonance spectroscopy to coronary artery disease. *Arterioscler Thromb Vasc Biol* 1998;18(7):1046-53.

169. Wang J, Stancakova A, Soininen P, et al. Lipoprotein subclass profiles in individuals with varying degrees of glucose tolerance: a population-based study of 9399 Finnish men. *J Intern Med* 2012;272(6):562-72.

170. Siri PW, Krauss RM. Influence of dietary carbohydrate and fat on LDL and HDL particle distributions. *Curr Atheroscler Rep* 2005;7(6):455-9.

171. Kontush A, Chapman MJ. Antiatherogenic small, dense HDL-guardian angel of the arterial wall? *Nat Clin Pract Cardiovasc Med* 2006;3(3):144-53.

172. Vaisar T, Pennathur S, Green PS, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Invest* 2007;117(3):746-56.

173. Liao F, Liu WL, Zhou QX, et al. Assay of serum arylesterase activity by fitting to the reaction curve with an integrated rate equation. *Clin Chim Acta* 2001;314(1-2):67-76.

174. Wallace TM, Levy JC, Matthews DR. Use and abuse of HOMA modeling. *Diabetes Care* 2004;27(6):1487-95.

175. Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955;34(9):1345-53.

176. Tcherkas YV, Denisenko AD. Simultaneous determination of several amino acids, including homocysteine, cysteine and glutamic acid, in human plasma by isocratic reversed-phase high-performance liquid chromatography with fluorimetric detection. *J Chromatogr A* 2001;913(1-2):309-13.

177. Lee MH, Hammad SM, Semler AJ, et al. HDL_3, but not HDL_2, stimulates plasminogen activator inhibitor-1 release from adipocytes: the role of sphingosine-1-phosphate. *J Lipid Res* 2010;51(9):2619-28.

178. Pedersen LR, Olsen RH, Frederiksen M, et al. Copenhagen study of overweight patients with coronary artery disease undergoing low energy diet or interval

training: the randomized CUT-IT trial protocol. *BMC Cardiovasc Disord* 2013;13:106.

179. Okajima F. Plasma lipoproteins behave as carriers of extracellular sphingosine-1-phosphate: is this an atherogenic mediator or an anti-atherogenic mediator? *Biochim Biophys Acta* 2002;1582(1-3):132-7.

180. Superko HR, Pendyala L, Williams PT, et al. High-density lipoprotein subclasses and their relationship to cardiovascular disease. *J Clin Lipidol* 2012;6(6):496-523.

181. Cotton PA, Subar AF, Friday JE, et al. Dietary sources of nutrients among US adults, 1994 to 1996. *J Am Diet Assoc* 2004;104(6):921-30.

182. Subar AF, Krebs-Smith SM, Cook A, et al. Dietary sources of nutrients among US adults, 1989 to 1991. *J Am Diet Assoc* 1998;98(5):537-47.

183. Mangravite LM, Chiu S, Wojnoonski K, et al. Changes in atherogenic dyslipidemia induced by carbohydrate restriction in men are dependent on dietary protein source. *J Nutr* 2011;141(12):2180-5.

184. Vessby B, Uusitupa M, Hermansen K, et al. Substituting dietary saturated for monounsaturated fat impairs insulin sensitivity in healthy men and women: the KANWU study. *Diabetologia* 2001;44(3):312-9.

185. Zivkovic AM, Wiest MM, Nguyen UT, et al. Effects of sample handling and storage on quantitative lipid analysis in human serum. *Metabolomics* 2009;5(4):507-16.

186. Caulfield MP, Li S, Lee G, et al. Direct determination of lipoprotein particle sizes and concentrations by ion mobility analysis. *Clin Chem* 2008;54(8):1307-16.

187. Maeda S, Nakanishi S, Yoneda M, et al. Associations between small dense LDL, HDL subfractions (HDL_2, HDL_3) and risk of atherosclerosis in Japanese-Americans. *J Atheroscler Thromb* 2012;19(5):444-52.

188. Perez-Mendez O, Torres-Tamayo M, Posadas-Romero C, et al. Abnormal HDL subclasses distribution in overweight children with insulin resistance or type 2 diabetes mellitus. *Clin Chim Acta* 2007;376(1-2):17-22.

189. Aoki S, Yatomi Y, Shimosawa T, et al. The suppressive effect of sphingosine 1-phosphate on monocyte-endothelium adhesion may be mediated by the

rearrangement of the endothelial integrins alpha(5)beta(1) and alpha(v)beta(3). *J Thromb Haemost* 2007;5(6):1292-301.

190. Storlien L, Oakes ND, Kelley DE. Metabolic flexibility. *Proc Nutr Soc* 2004;63(02):363-8.

191. Xu N, Dahlback B. A novel human apolipoprotein (apoM). *J Biol Chem* 1999;274(44):31286-90.

192. Christoffersen C, Pedersen TX, Gordts PL, et al. Opposing effects of apolipoprotein m on catabolism of apolipoprotein B-containing lipoproteins and atherosclerosis. *Circ Res* 2010;106(10):1624-34.

193. Verhoef P, Stampfer MJ, Buring JE, et al. Homocysteine metabolism and risk of myocardial infarction: relation with vitamins B6, B12, and folate. *Am J Epidemiol* 1996;143(9):845-59.

194. Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. Washington, DC: Natl Acad Press 1998;8:197-305.

195. Cole CB, Nikpay M, Lau P, et al. Adiposity significantly modifies genetic risk for dyslipidemia. *J Lipid Res* 2014;55(11):2416-22.

196. Holt R, Yim SJ, Shearer G, et al. Correlation of lipoprotein epoxide content to microvascular function after short-term walnut intake. *Faseb J* 2014;28(1):S831.5.

197. Strassburg K, Esser D, Vreeken RJ, et al. Postprandial fatty acid specific changes in circulating oxylipins in lean and obese men after high-fat challenge tests. *Mol Nutr Food Res* 2013;58(3):591-600.

198. Keenan AH, Pedersen TL, Fillaus K, et al. Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers. *J Lipid Res* 2012;53(8):1662-9.

199. Newman JW, Pedersen TL, Brandenburg VR, et al. Effect of omega-3 fatty acid ethyl esters on the oxylipin composition of lipoproteins in hypertriglyceridemic, statin-treated subjects. *PloS One* 2014;9(11):e111471.

200. Michaelson LV, Zauner S, Markham JE, et al. Functional characterization of a higher plant sphingolipid Delta4-desaturase: defining the role of sphingosine and sphingosine-1-phosphate in Arabidopsis. *Plant Physiol* 2009;149(1):487-98.

201. Shannon P, Markiel A, Ozier O, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 2003;13(11):2498-504.

202. Erlemann KR, Cossette C, Grant GE, et al. Regulation of 5-hydroxyeicosanoid dehydrogenase activity in monocytic cells. *Biochem J* 2007;403(1):157-65.

203. Grant GE, Rokach J, Powell WS. 5-oxo-ETE and the OXE receptor. *Prostaglandins Other Lipid Mediat* 2009;89(3-4):98-104.

204. Powell WS, Chung D, Gravel S. 5-oxo-6,8,11,14-eicosatetraenoic acid is a potent stimulator of human eosinophil migration. *J Immunol* 1995;154(8):4123-32.

205. Navab M, Ananthramaiah GM, Reddy ST, et al. The oxidation hypothesis of atherogenesis: the role of oxidized phospholipids and HDL. *J Lipid Res* 2004;45(6):993-1007.

206. Bowry VW, Stanley KK, Stocker R. High-density lipoprotein is the major carrier of lipid hydroperoxides in human blood-plasma from fasting donors. *Proc Natl Acad Sci USA* 1992;89(21):10316-20.

207. Morisseau C, Hammock BD. Impact of soluble epoxide hydrolase and epoxyeicosanoids on human health. *Annu Rev Pharmacol Toxicol* 2013;53:37-58.

208. Lee JP, Yang SH, Lee HY, et al. Soluble epoxide hydrolase activity determines the severity of ischemia-reperfusion injury in kidney. *PloS One* 2012;7(5):e37075.

209. Slim R, Hammock BD, Toborek M, et al. The role of methyl-linoleic acid epoxide and diol metabolites in the amplified toxicity of linoleic acid and polychlorinated biphenyls to vascular endothelial cells. *Toxicol Appl Pharmacol* 2001;171(3):184-93.

210. Viswanathan S, Hammock BD, Newman JW, et al. Involvement of CYP 2C9 in mediating the proinflammatory effects of linoleic acid in vascular endothelial cells. *J Am Coll Nutr* 2003;22(6):502-10.

211. Keenan AH, Pedersen TL, Fillaus K, et al. Basal omega-3 fatty acid status affects fatty acid and oxylipin responses to high-dose n3-HUFA in healthy volunteers. *J Lipid Res* 2012;53(8):1662-9.

212. Schuchardt JP, Schmidt S, Kressel G, et al. Modulation of blood oxylipin levels by long-chain omega-3 fatty acid supplementation in hyper- and normolipidemic men. *Prostaglandins Leukot Essent Fatty Acids* 2014;90(2-3):27-37.

213. Bouwens M, van de Rest O, Dellschaft N, et al. Fish-oil supplementation induces anti-inflammatory gene expression profiles in human blood mononuclear cells. *Am J Clin Nutr* 2009;90(2):415-24.

214. Schebb NH, Ostermann AI, Yang J, et al. Comparison of the effects of long-chain omega-3 fatty acid supplementation on plasma levels of free and esterified oxylipins. *Prostaglandins Other Lipid Mediat* 2014;113-115:21-9.

215. Schuchardt JP, Schneider I, Willenberg I, et al. Increase of EPA-derived hydroxy, epoxy and dihydroxy fatty acid levels in human plasma after a single dose of long-chain omega-3 PUFA. *Prostaglandins Other Lipid Mediat* 2014;109:23-31.

216. Zhou L, Nilsson A. Sources of eicosanoid precursor fatty acid pools in tissues. *J Lipid Res* 2001;42(10):1521-42.

217. Nelson GJ, Schmidt PC, Bartolini G, et al. The effect of dietary arachidonic acid on plasma lipoprotein distributions, apoproteins, blood lipid levels, and tissue fatty acid composition in humans. *Lipids* 1997;32(4):427-33.

218. La Belle M, Krauss RM. Differences in carbohydrate content of low density lipoproteins associated with low density lipoprotein subclass patterns. *J Lipid Res* 1990;31(9):1577-88.

219. Chait A, Brazg RL, Tribble DL, et al. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *Am J Med* 1993;94(4):350-6.

220. Nigon F, Lesnik P, Rouis M, et al. Discrete subspecies of human low density lipoproteins are heterogeneous in their interaction with the cellular LDL receptor. *J Lipid Res* 1991;32(11):1741-53.

221. St-Pierre AC, Ruel IL, Cantin B, et al. Comparison of various electrophoretic characteristics of LDL particles and their relationship to the risk of ischemic heart disease. *Circulation* 2001;104(19):2295-9.

222. Dreon DM, Fernstrom HA, Miller B, et al. Apolipoprotein E isoform phenotype and LDL subclass response to a reduced-fat diet. *Arterioscler Thromb Vasc Biol* 1995;15(1):105-11.

223. Dreon DM, Fernstrom HA, Williams PT, et al. Reduced LDL particle size in children consuming a very-low-fat diet is related to parental LDL-subclass patterns. *Am J Clin Nutr* 2000;71(6):1611-6.

224. Kasim-Karakas SE, Lane E, Almario R, et al. Effects of dietary fat restriction on particle size of plasma lipoproteins in postmenopausal women. *Metabolism* 1997;46(4):431-6.

225. Krauss RM, Blanche PJ, Rawlings RS, et al. Separate effects of reduced carbohydrate intake and weight loss on atherogenic dyslipidemia. *Am J Clin Nutr* 2006;83(5):1025-31.

226. Krauss RM, Dreon DM. Low-density lipoprotein subclasses and response to a low-fat diet in healthy men. *Am J Clin Nutr* 1995;62(2):S478-87.

227. Furtado JD, Campos H, Appel LJ, et al. Effect of protein, unsaturated fat, and carbohydrate intakes on plasma apolipoprotein B and VLDL and LDL containing apolipoprotein C-III: results from the OmniHeart Trial. *Am J Clin Nutr* 2008;87(6):1623-30.

228. Desroches S, Mauger JF, Ausman LM, et al. Soy protein favorably affects LDL size independently of isoflavones in hypercholesterolemic men and women. *J Nutr* 2004;134(3):574-9.

229. Greaves KA, Parks JS, Williams JK, et al. Intact dietary soy protein, but not adding an isoflavone-rich soy extract to casein, improves plasma lipids in ovariectomized cynomolgus monkeys. *J Nutr* 1999;129(8):1585-92.

230. Roussell MA, Hill AM, Gaugler TL, et al. Effects of a DASH-like diet containing lean beef on vascular health. *J Hum Hypertens* 2014;28:600-605.

231. Kwiterovich PO. Clinical relevance of the biochemical, metabolic, and genetic factors that influence low-density lipoprotein heterogeneity. *Am J Cardiol* 2002;90(8A):30i-47i.

232. Heinze CR, Hawkins MG, Gillies LA, et al. Effect of dietary omega-3 fatty acids on red blood cell lipid composition and plasma metabolites in the cockatiel, Nymphicus hollandicus. *J Anim Sci* 2012;90(9):3068-79.

233. Berneis KK, Krauss RM. Metabolic origins and clinical significance of LDL heterogeneity. *J Lipid Res* 2002;43(9):1363-79.

234. Dumesnil JG, Turgeon J, Tremblay A, et al. Effect of a low-glycaemic index-low-fat-high protein diet on the atherogenic metabolic risk profile of abdominally obese men. *Brit J Nutr* 2001;86(5):557-68.

235. Packard CJ, Demant T, Stewart JP, et al. Apolipoprotein B metabolism and the distribution of VLDL and LDL subfractions. *J Lipid Res* 2000;41(2):305-17.

236. Lefevre M, Champagne CM, Tulley RT, et al. Individual variability in cardiovascular disease risk factor responses to low-fat and low-saturated-fat diets in men: body mass index, adiposity, and insulin resistance predict changes in LDL cholesterol. *Am J Clin Nutr* 2005;82(5):957-63.

237. Schaefer EJ, Lichtenstein AH, Lamon-Fava S, et al. Efficacy of a National Cholesterol Education Program Step 2 diet in normolipidemic and hypercholesterolemic middle-aged and elderly men and women. *Arterioscler Thromb Vasc Biol* 1995;15(8):1079-85.

238. Lyons TJ. Lipoprotein glycation and its metabolic consequences. *Diabetes* 1992;41:67-73.

239. Younis NN, Soran H, Pemberton P, et al. Small dense LDL is more susceptible to glycation than more buoyant LDL in type 2 diabetes. *Clin Sci* 2013;124(5-6):343-9.

240. Berglund L, Oliver EH, Fontanez N, et al. HDL-subpopulation patterns in response to reductions in dietary total and saturated fat intakes in healthy subjects. *Am J Clin Nutr* 1999;70(6):992-1000.

241. Brinton EA, Eisenberg S, Breslow JL. A low-fat diet decreases high density lipoprotein (HDL) cholesterol levels by decreasing HDL apolipoprotein transport rates. *J Clin Invest* 1990;85(1):144-51.

242. Hayek T, Ito Y, Azrolan N, et al. Dietary fat increases high density lipoprotein (HDL) levels both by increasing the transport rates and decreasing the fractional catabolic rates of HDL cholesterol ester and apolipoprotein (Apo) A-I. Presentation of a new animal model and mechanistic studies in human Apo A-I transgenic and control mice. *J Clin Invest* 1993;91(4):1665-71.

243. Brinton EA, Eisenberg S, Breslow JL. Human HDL cholesterol levels are determined by apoA-I fractional catabolic rate, which correlates inversely with estimates of HDL particle size. Effects of gender, hepatic and lipoprotein lipases, triglyceride and insulin levels, and body fat distribution. *Arterioscler Thromb Vasc Biol* 1994;14(5):707-20.

244. Jensen MK, Rimm EB, Furtado JD, et al. Apolipoprotein C-III as a potential modulator of the association between HDL-cholesterol and incident coronary heart disease. *J Am Heart Assoc* 2012;1(2):jah3-e000232.